

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 13-44406- -4	FECHA: 2013-12-27 08:52:49
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 27
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
GERMAN ALONSO CUBILLOS MENDOZA

Asunto: Radicación: 13-44406- -4
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 23667
 Folios: 27

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/JP2011/071174				
Fecha de Presentación Internacional	12/09/2011				

1. Documentos estudiados

Descripción:	Radicado N°13-044406-00000-0000	Marzo 6 de 2013
Reivindicaciones	Radicado N°13-044406-00000-0000	Marzo 6 de 2013
Prioridad:	JP 2010 – 204747	Septiembre 13 de 2010

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

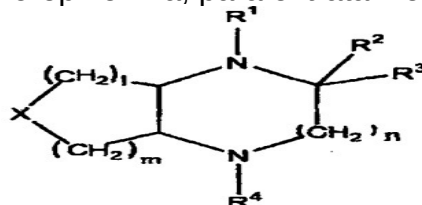
Por lo tanto, las reivindicaciones 1 a 16 del Radicado N°13-044406-00000-0000 se aceptan para ser estudiadas en su totalidad, porque se incluye el pago por las

reivindicaciones adicionales 11 a 16, de acuerdo con el recibo presente en la página 4 del Radicado N°13-044406-00000-0000.

Título de la solicitud: “Compuestos heterocíclicos para tratar o prevenir trastornos causados por neurotransmisión reducida de Serotonina, Norepinefrina o Dopamina”

El problema planteado en esta solicitud consiste en la obtención de compuestos alternativos para el tratamiento de la depresión.

Para resolver este problema técnico, la solicitud en estudio plantea la obtención de compuestos heterocíclicos que actúan contra la neurotransmisión reducida de Dopamina, Serotonina y Norepinefrina, para el tratamiento de la depresión.



El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07D241/38; C07D243/10; C07D401/04; C07D403/04; C07D403/10; C07D405/04; C07D405/10; C07D409/04; C07D409/10; C07D413/10; C07D417/04; C07D417/10; C07D471/04; C07D491/04; C07D495/04.

4. Excepción a la Patentabilidad (Art 20 D 486, literal d)

El objeto de las reivindicación 15 no es patentable, de acuerdo con el artículo citado.

5. Reivindicaciones relativas al uso o a un segundo uso (Arts. 14 y 21 D 486)

El uso de las reivindicaciones 8 a 14 no es patentable, de acuerdo con el artículo 14.

6. De la Solicitud de la Patente

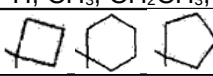
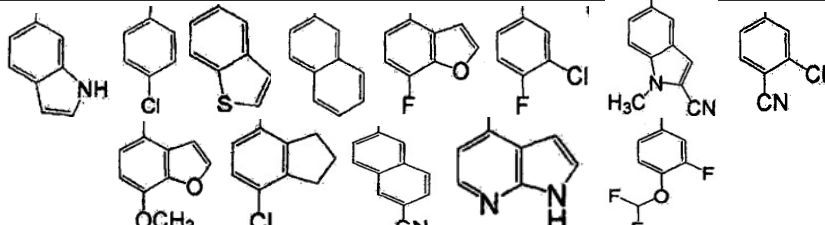
Título

El título de la presente solicitud se considera muy general y ambiguo, porque al mencionar “compuestos heterocíclicos” existe una infinidad de compuestos que son heterocíclicos pero que no hacen parte del objeto de la presente invención. Se observa que los compuestos de la presente invención son derivados de decahidrobenzoquinoxalina, derivados de octahidrociclopentanpirazina y derivados de hexahidrofuro [3,4-b pirazina]. Se solicita modificar el título de la presente invención de acuerdo con las observaciones anteriores.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicación 1 no es concisa, porque se mencionan sustituyentes mencionados de manera ambigua tales como “alquilo inferior, hidroxialquilo inferior, alcoxi inferior, alquiltio inferior, alquilamino inferior, alquenilfenilo inferior grupo protector, trialquilsiloxi alquilo inferior”, así como se mencionan sustituyentes de manera general como “heterociclo con sustituyentes arbitrarios”, dado que a partir de los mismos se obtiene una cantidad indefinida de posibles compuestos, de tal manera que se incrementa la complejidad de la presente solicitud y por lo tanto, no es posible definir de forma certera el verdadero alcance de lo que se desea proteger. Por lo tanto, se solicita realizar una restricción más razonable de los sustituyentes de la presente invención, de tal forma que a partir de estos se

obtingan los compuestos sintetizados, probados y que representan la solución al problema técnico planteado dentro de la presente invención. Se solicita mencionar específicamente los sustituyentes empleados (por ejemplo, en vez de mencionar alquilo inferior, mencionar si es metil, etil, isopropil, butil, pentil; en vez de mencionar heterociclo, mencionar si es furano, tiofeno, pirrolidina, carbazol, piridina, tiazol, benzofurano, quinazolina, entre otros; así mismo con los demás sustituyentes). Es cierto que un inventor esta en el pleno derecho de proteger su invención en la extensión que desee siempre y cuando se conserve un único concepto inventivo, sin embargo, el artículo 30 de la Decisión 486 determina que las reivindicaciones que definen una invención deben ser claras, concisas y estar plenamente detalladas en el capítulo descriptivo. Se sugiere al solicitante realizar dicha restricción, de acuerdo a lo que se muestra en la siguiente tabla:

Sustituyente	Soportado en la descripción
X	CH ₂ , O
(CH ₂) _l , (CH ₂) _m y (CH ₂) _n	l, m y n son 1 ó 2
R ¹	H, CH ₃ , CH ₂ CH ₂ OH, 
R ² y R ³	H, CH ₃ , CH ₂ CH ₃ ,
R ² + R ³	
R ⁴	

De igual manera, se solicita realizar las mismas restricciones para las reivindicaciones 2 a 7, de acuerdo a las observaciones realizadas para la reivindicación 1

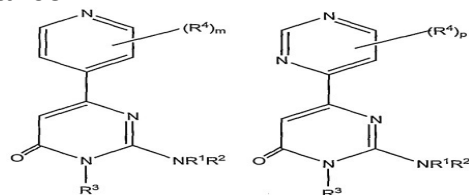
Para la reivindicación 16, se solicita mencionar de manera específica los reactivos y las condiciones de reacción involucradas en la síntesis de los compuestos de la presente invención.

7. Determinación del Estado de la Técnica

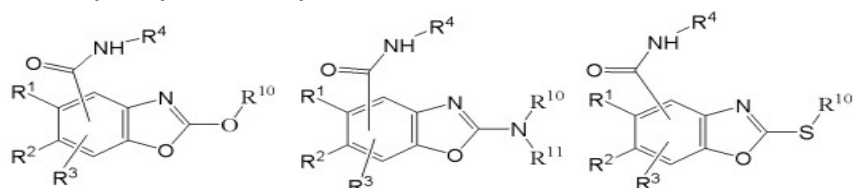
El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de la cual se invoca prioridad, es decir, el 13 de septiembre de 2010. Consultadas las fuentes de información con que se cuenta, se encontró los siguientes documentos que anticipan el objeto de esta solicitud.

Item	Documento	Título	Fecha de Publicación
D1	WO2008023239	"Pyrimidone compounds as GSK – 3 inhibitors"	Febrero 28 de 2008
D2	WO2008019372	"2 – aminobenzoxazole carboxamides as 5 – HT modulators"	Febrero 14 de 2008
D3	WO2004056784	"Novel carboxamides, the production and use thereof as medicaments"	Julio 8 de 2004
D4	WO0067735	"Use of selective antagonists of the α _{1b} adrenergic receptor for improvement of sexual dysfunction"	Noviembre 16 de 2000
D5	ES2140565	"Nuevos derivados de pirazina carboxamida, su preparación y su utilización en medicamentos"	Agosto 21 de 1996
D6	<i>Journal of Medicinal Chemistry</i> . 2009. 52; 4951 – 4954	"Doxazosin – Related α ₁ Adrenoreceptor Antagonist With Prostate Antitumor Activity "	Febrero 7 de 2009
D7	<i>Bioorganic & Medicinal Chemistry</i> . 2007. 15; 2334 – 2345	"Synthesis and α ₁ adrenoreceptor antagonist activity of derivatives and isósters of the furan portion of (+) cyclazosin"	Enero 19 de 2007

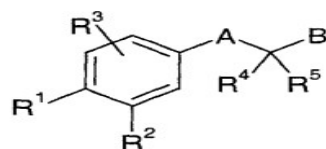
El documento D1¹ divulga compuestos derivados de 4 – (piridin – 4 – il/pirimidin – 4 – il) – 2 – aminopirimidin – 4 - ona, como inhibidores de la Protein Kinasa GSK – 3, para el tratamiento del cáncer.



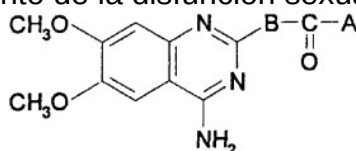
El documento D2² divulga compuestos derivados de benzoxazol – 4 – il carboxamida, como inhibidores de 5 – HT₃, para el tratamiento de las náuseas y el vómito inducidos por quimioterapia



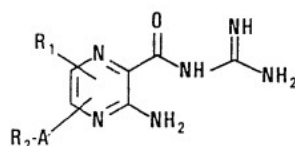
El documento D3³ divulga compuestos de fenil carboxamidas con actividad antitrombotica



El documento D4⁴ divulga compuestos derivados de 4 – amino – 6, 7 – dimetoxi Quinazolina, para el tratamiento de la disfunción sexual



El documento D5⁵ divulga compuestos derivados de 3 – aminopirazin – 2 – (N – guanidinio) carboxamida como agentes para el tratamiento de isquemias



El documento D6⁶ divulga compuestos derivados de Doxazosina como inhibidores del receptor α₁, para el tratamiento de BPH y cáncer de próstata

1http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2008023239A1&KC=A1&FT=D&ND=3&date=20080228&DB=worldwide.espacenet.com&locale=es_LP

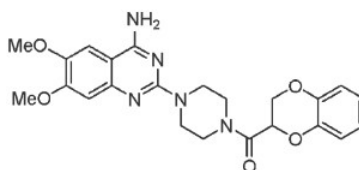
2http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2008019372A2&KC=A2&FT=D&ND=3&date=20080214&DB=worldwide.espacenet.com&locale=es_LP

3http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=2004056784A1&KC=A1&FT=D&ND=3&date=20040708&DB=worldwide.espacenet.com&locale=es_LP

4http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=0067735A2&KC=A2&FT=D&ND=3&date=20001116&DB=worldwide.espacenet.com&locale=es_LP

5http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=ES&NR=2140565T3&KC=T3&FT=D&ND=3&date=20000301&DB=worldwide.espacenet.com&locale=es_LP

6<http://pubs.acs.org/doi/abs/10.1021/jm8016046>

COC1=CC=C2C(=C1)N=C(N)N=C2N3CC[C@H]4[C@@H](C(=O)C5=CC=CC5O)CC[C@H]3C4

Novedad (Art 16 D 486)

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D1
Compuesto de la fórmula I R^2 y R^3 son H, $(CH_2)_n$, donde n es 1 X es CH_2, $(CH_2)_l$, donde l es 1; $(CH_2)_m$, donde m es 2	Ejemplo 25
R^1 es un grupo protector	Grupo Boc (tercbutoxicarbonilo)
R^4 es un grupo heterocíclico con sustituyentes arbitrarios	

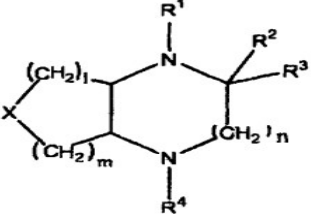
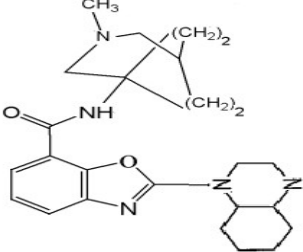
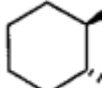
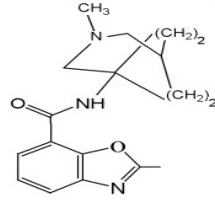
En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D1, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

7<http://www.sciencedirect.com/science/article/pii/S0968089607000387>

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D2
Compuesto de la fórmula I  R¹, R² y R³ son H, (CH₂)_n, donde n es 1	Ejemplo 126 
X es CH₂, (CH₂)_l, donde l es 1; (CH₂)_m, donde m es 2	
R⁴ es un grupo heterocíclico con sustituyentes arbitrarios	

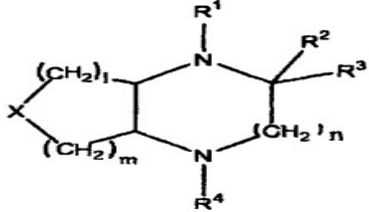
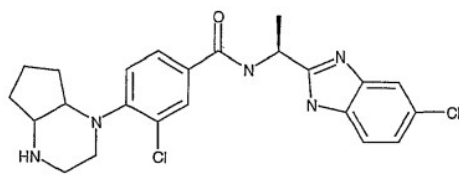
Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D2 (D2: Página 123, ejemplo 126), en donde se muestra el compuesto Endo – N – (9 – metil – 9 – azabiclo – [3.3.1] nonan – 3 – il) – 2 – ((4aS, 8aS) – octahidroquinoxalin – 1(2H) – il) benzoxazol – 4 - carboxamida, como inhibidor de 5HT₃ para el tratamiento de las nauseas y el vómito inducidos por la quimioterapia, con las características técnicas esenciales descritas en la tabla anterior.

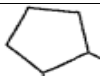
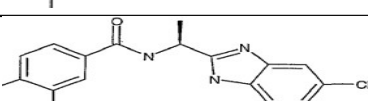
En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D2, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D3
Compuesto de la fórmula I  R¹, R² y R³ son H, (CH₂)_n, donde n es 1	Ejemplo 397 

X es CH ₂ , (CH ₂) _l , donde l es 1; (CH ₂) _m , donde m es 1	
R ⁴ es un grupo heterocíclico con sustituyentes arbitrarios	

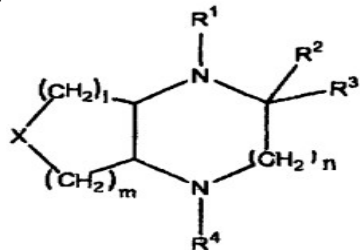
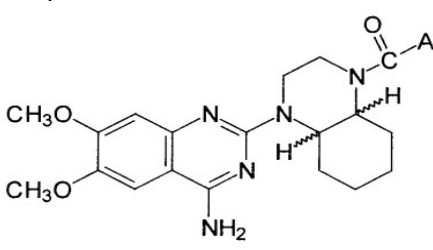
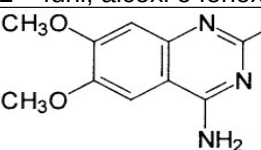
Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D3 (D3: Página 398, ejemplo 397), en donde se muestra el compuesto 3 – cloro – N – [(1S) – 1 – (5 – cloro – 1H – bencimidazol – 2 – il) etil] – 4 – (2, 3 – dioxo octahidrociclopentapirazin – 1 – il) benzamida, como un agente antitrombótico, con las características técnicas esenciales descritas en la tabla anterior.

En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D3, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D4
Compuesto de la fórmula I  R ² y R ³ son H, (CH ₂) _n , donde n es 1 X es CH ₂ , (CH ₂) _l , donde l es 1; (CH ₂) _m , donde m es 2	Compuesto de la fórmula II 
R ¹ es un grupo protector	CO – A, donde A es 2 – tetrahydrofuril, 2 – furil, alcoxi o fenoxialquil
R ⁴ es un grupo heterocíclico con sustituyentes arbitrarios	

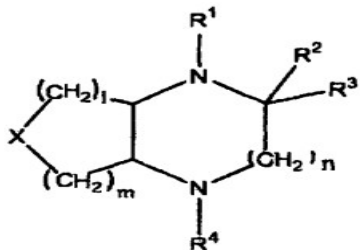
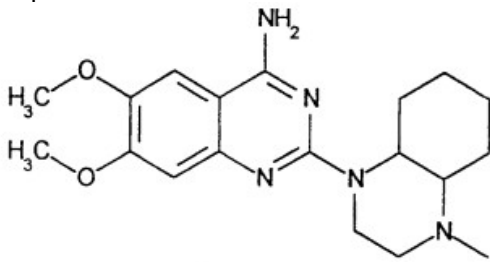
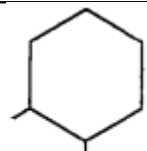
Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D4 (D4: página 5, líneas 4 – 8), en donde se muestran compuestos derivados de 4 – amino – 6, 7 – dimetoxi quinazolina, para el tratamiento de la disfunción sexual, con las características técnicas esenciales descritas en la tabla anterior. Nótese que la expresión “grupo protector” es muy general y ambigua, por lo cual cualquier estructura química susceptible a ser reemplazada en una reacción química, se considera un grupo protector.

En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D4, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D5
Compuesto de la fórmula I  R ² y R ³ son H, (CH ₂) _n , donde n es 1 X es CH ₂ , (CH ₂) _l , donde l es 1; (CH ₂) _m , donde m es 2	Compuesto de fórmula XIII 
R ¹ es alquilo inferior	Grupo metil en posición 4 del anillo de octahidrobenczoquinoxalina
R ⁴ es un grupo heterocíclico con sustituyentes arbitrarios	

Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D5 (D5: Página 9, compuesto XIII) en donde se muestra 4 – amino – 6, 7 – dimetoxi quinazolina, para el tratamiento de la disfunción sexual, con las características técnicas esenciales descritas en la tabla anterior.

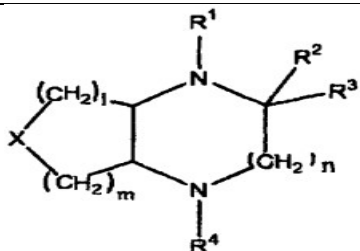
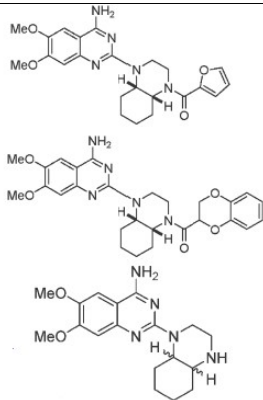
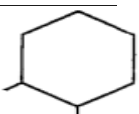
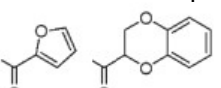
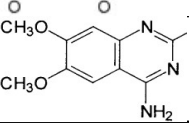
En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D5, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D6
Compuesto de la fórmula I	Compuestos:

 R² y R³ son H, (CH₂)_n, donde n es 1	
X es CH₂, (CH₂)_l, donde l es 1; (CH₂)_m, donde m es 2	
R¹ es H o un grupo protector	En posición 4 del anillo de octahidrobenzoquinoxalina esta H o 
R⁴ es un grupo heterocíclico con sustituyentes arbitrarios	

Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D6 (D6: Página 4952, figura 1, compuestos 1, 1a, 2, ciclazosin y 5, 6, 7) en donde se muestran compuestos derivados de 4 – amino – 6, 7 – dimetoxi quinazolina,, como inhibidores del receptor 1 para el tratamiento de la HPB o del cáncer de próstata, con las características técnicas esenciales descritas en la tabla anterior. Nótese que la expresión “grupo protector” es muy general y ambigua, por lo cual cualquier estructura química susceptible a ser reemplazada en una reacción química, se considera un grupo protector.

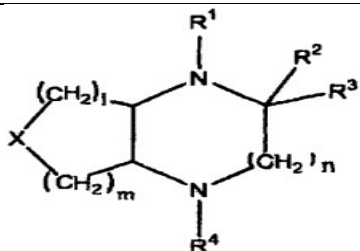
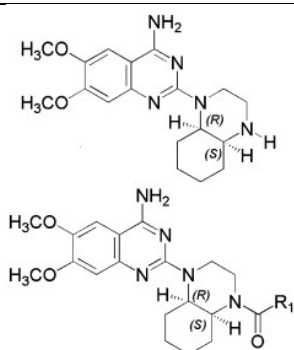
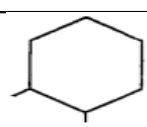
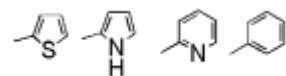
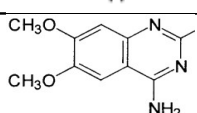
En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D6, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

La reivindicación 1 divulga un compuesto heterocíclico representado por la fórmula general I o una sal del mismo, caracterizados porque... (Página 386 del radicado N°13-044406-00000-0000).

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Solicitud	D7
Compuesto de la fórmula I	Compuestos

 R^2 y R^3 son H, $(CH_2)_n$, donde n es 1	
X es CH_2, $(CH_2)_l$, donde l es 1; $(CH_2)_m$, donde m es 2	
R^1 es H o un grupo protector	En posición 4 del anillo de octahidrobenzoquinoxalina esta H o R^1, donde R^1 es: 
R^4 es un grupo heterocíclico con sustituyentes arbitrarios	

Las características técnicas esenciales de los compuestos de la fórmula I de acuerdo con la reivindicación 1, se han divulgado previamente en D7 (Página 2335, compuestos 2 a 9 y 12) en donde se muestran compuestos derivados de 4 – amino – 6, 7 – dimetoxi quinazolina,, como inhibidores del receptor 1 para el tratamiento de la HPB o del cáncer de próstata, con las características técnicas esenciales descritas en la tabla anterior. Nótese que la expresión “grupo protector” es muy general y ambigua, por lo cual cualquier estructura química susceptible a ser reemplazada en una reacción química, se considera un grupo protector.

En consecuencia, la reivindicación 1, de acuerdo a lo divulgado en D7, carece de Novedad.

Las reivindicaciones 2, 3 y 7, dependientes de la reivindicación 1, no divulgan características adicionales que otorguen novedad a los compuestos de la reivindicación 1. Por lo tanto, estas reivindicaciones también carecen de Novedad.

8. Conclusión

Los requisitos de Patentabilidad de esta solicitud se ven afectados de la siguiente manera:

Item	Documento	Reivindicaciones	Requisitos que afecta
D1	WO2008023239	1, 2, 3 y 7	Novedad
D2	WO2008019372	1, 2, 3 y 7	Novedad
D3	WO2004056784	1, 2, 3 y 7	Novedad
D4	WO0067735	1, 2, 3 y 7	Novedad
D5	ES2140565	1, 2, 3 y 7	Novedad
D6	<i>Journal of Medicinal Chemistry.</i> 2009. 52; 4951 – 4954	1, 2, 3 y 7	Novedad
D7	<i>Bioorganic & Medicinal Chemistry.</i> 2007. 15; 2334 – 2345	1, 2, 3 y 7	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-12-31

Elaboró: Dayron Alexander Ramirez Reyes
Revisó: Gloria Jacqueline Alfonso Roza

Doxazosin-Related α_1 -Adrenoceptor Antagonists With Prostate Antitumor Activity

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Received December 18, 2008

Doxazosin analogues **1–3** and **1a** were synthesized and investigated at α_1 -adrenoceptors and PC-3, DU-145, and LNCaP human prostate cancer cells. Compound **1** (cyclodoxazosin) was a potent α_{1B} -adrenoceptor antagonist displaying antiproliferative activity higher than that of doxazosin in cancer cells in vitro and in vivo, respectively. Because of its antitumor efficacy at low concentrations, lower apoptotic activity in NHDF vs tumor cells, and antiangiogenic effect, **1** showed a better therapeutic profile relative to doxazosin.

Introduction

Doxazosin is a potent and selective α_1 -adrenoceptor antagonist displaying effective antihypertensive activity.¹ Because of its relaxant activity and increased apoptosis on prostate smooth muscle,^{2,3} it is also used to treat the obstructive and irritative symptoms of BPH^a disease.⁴ Doxazosin exerts, via an apoptotic-induced mechanism, a potent anti-growth effect in vitro against the PC-3, DU-145, and LNCaP human prostate cancer cells, independently of its α_1 -adrenoceptor antagonism or the hormone sensitivity status of cells.^{5,6} The antitumor activity of doxazosin was confirmed in mice bearing PC-3-induced prostate cancer, where it displayed a significant inhibition of tumor growth.⁵ These properties point to a therapeutic significance for the quinazoline-based compounds both in managing human BPH and in the anti-tumor treatment of prostate cancer,⁶ with a possible role in the prevention of tumor development.⁷

In our continuing search for quinazoline-based α_1 -adrenoceptor antagonists, we developed cyclazosin, a racemic compound known to possess high antagonist affinity and selectivity for α_{1A} - and α_{1B} -adrenoceptors relative to the α_{1A} subtype.^{8,9} Here, we report on two novel cyclazosin analogues, 2-[4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)-*cis*-octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine (**1**, cyclodoxazosin) and 2-[4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)-*trans*-octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine (**2**), incorporating a 2,3-dihydro-1,4-benzodioxine-2-carbonyl moiety instead of the 2-furoyl group, and on the related, previously described,¹⁰ open analogue *N*-{6-[(4-amino-6,7-dimethoxyquinazolin-2-yl) (methyl)amino]hexyl}-*N*-methyl-2,3-dihydro-1,4-benzodioxine-2-carboxamide (**3**), in order to assess the role, if any, of the

decahydroquinoxaline nucleus on the α_1 -adrenoceptor subtypes antagonism and the antitumor activity. In addition, because **1** is more potent than the relative *trans* isomer **2**, to evaluate the effect of the stereochemistry of the *cis*-octahydroquinoxalin nucleus on the antiproliferative activity (–)-2-[4a*S*,8a*R*]-4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)-octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine (**1a**), a couple of the four stereoisomers of **1**, was included in the study. The stereochemistry of the 1,4-benzodioxine moiety was not investigated because doxazosin was less potent than **1**. The structures of the studied compounds are reported in Figure 1.

Results and Discussion

Chemistry. The synthesis of compounds **1** and **2** was achieved by reaction of 2,3-dihydro-1,4-benzodioxine-2-carbonyl chloride (**4**)¹¹ with *cis*-(**5**)¹² or *trans*-6,7-dimethoxy-2-[octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine (**7**), obtained from 2-chloro-6,7-dimethoxyquinazolin-4-amine and *cis*- or *trans*-decahydroquinoxaline,¹³ respectively (Scheme 1). Similarly, **1a** was obtained from **4** and (–)-6,7-dimethoxy-2-[4a*S*,8a*R*]-octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine (**6**).¹⁴ Compound **3** was obtained by reaction of **4** with 6,7-dimethoxy-*N*-methyl-*N*-[6-(methylamino)hexyl]quinazoline-2,4-diamine, as reported.¹⁰ Compounds **1** and **2** are mixtures of four stereoisomers because of the three chiral centers present in their structures. Compound **1** was isolated as mixture of two racemates, in a proportion of 28% and 72%, as shown by the HPLC/DAD analysis, whereas **2** was not analyzed. (see Supporting Information).

α_1 -Adrenoceptor Antagonist Activity and Binding Affinity. Compounds **1–3** and cyclazosin were studied in rat isolated prostatic vas deferens, aorta, and spleen to assess their antagonist activity and selectivity at α_1 -adrenoceptor subtypes.^{15–17} The antagonist potency of cyclazosin at α_{1A} , α_{1B} , and α_{1D} subtypes and of **1** at the α_{1D} subtype was expressed as pA_2 values, according to Arunlakshana and Schild.¹⁸ For **2**, **3**, and for **1** at α_{1A} , and α_{1B} adrenoceptors, as the slope of

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^aAbbreviations: BPH, benign prostatic hyperplasia; NHDF, normal human dermal fibroblasts.

the Schild plot was significantly different from unity, the potency was expressed by pK_B values, according to van Rossum.¹⁹ In this latter case, the pK_B value was calculated at the lowest antagonist concentration, giving a significant rightward shift of the agonist concentration–response curve [$\log(\text{concentration ratio} - 1) \geq 0.5$].

The affinity profile of **1–3** and **1a** was also evaluated in radioreceptor binding assays on membranes of Chinese hamster ovary (CHO) cells expressing human α_{1A} , α_{1B} , and α_{1D} adrenoceptor subtypes.²⁰ Binding affinities were expressed as pK_i values derived using the Cheng–Prusoff equation.²¹ The results are reported in Table 1 in comparison with cyclazosin and doxazosin, the reference compounds.

In functional assays, **3** was more potent than **1** and **2** at all α_1 -adrenoceptor subtypes with the exception of **1** at the α_{1B} subtype. Clearly, the insertion of the piperazine ring

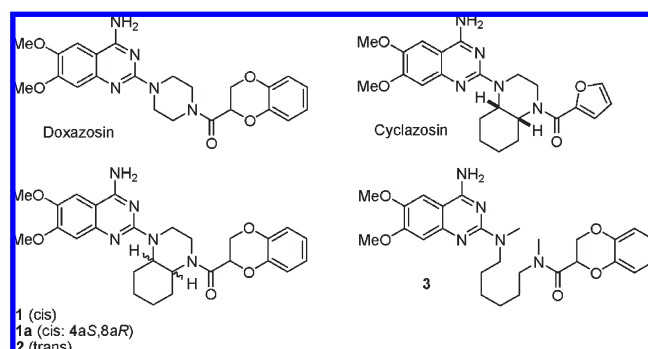
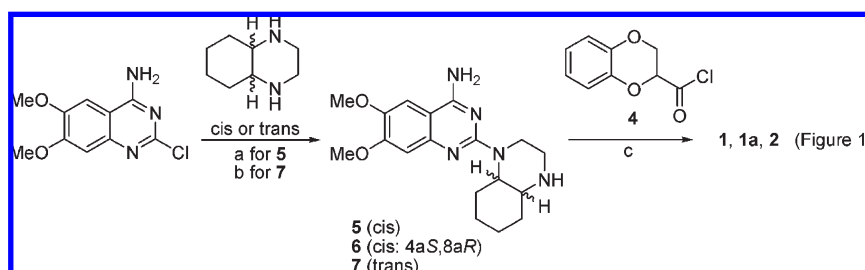


Figure 1. Chemical structures of doxazosin, cyclazosin, **1–3**, and **1a**.

Scheme 1^a



^a Reagents: (a) *i*-AmOH, reflux; (b) *i*-AmOH, triethylamine, DMAP, reflux; (c) dry CH_2Cl_2 .

Table 1. Binding (pK_i) and Functional Affinity (pA_2 or pK_B) Constants and in Vitro Antiproliferative Activity (IC_{50}) of **1–3**, **1a**, Cyclazosin, and Doxazosin at Cloned Human α_1 -Adrenoceptors, Isolated Rat Tissues α_1 -Adrenoceptors, and PC-3, DU-145, and LNCaP Human Prostate Cancer Cells and NHDF Cells^a

no.	pK_i ^a			pA_2 ^b or pK_B ^c			IC_{50} (μM) ^d			
	α_{1A}	α_{1B}	α_{1D}	α_{1A}	α_{1B}	α_{1D}	PC-3	DU-145	LNCaP	NHDF
1	8.55	9.38	9.77	7.21 ± 0.07^c	8.30 ± 0.05^c	7.39 ± 0.06^b	0.85	0.96	0.57	1.46
1a	8.47	8.85	9.31				1.02			
2	7.09	7.65	7.58	5.88 ± 0.18^c	7.14 ± 0.07^c	6.88 ± 0.10^c	$> 10^g$	$> 10^g$	9.75	$> 10^g$
3	8.73	9.03	8.80	8.51 ± 0.07^c	8.35 ± 0.10^c	8.64 ± 0.13^c	8.40	9.25	4.61	9.80
cyclazosin	8.26 ^e	9.49 ^e	9.77 ^e	8.91 ± 0.04^b	9.18 ± 0.03^b	9.21 ± 0.02^b				
doxazosin	9.27 ^f	9.09 ^f	9.09 ^f	$8.69 \pm 0.70^{b,f}$	$9.51 \pm 0.41^{b,f}$	$8.97 \pm 0.23^{b,f}$	38.60	37.44	28.11	43.00

^a Equilibrium dissociation constants (pK_i) were calculated from IC_{50} values using the Cheng–Prusoff equation. The affinity estimates, derived from displacement of [^3H]prazosin binding from α_1 -adrenoceptors, were from two to three experiments performed in triplicate, which agreed within $\pm 20\%$.

^b The α_1 -adrenoceptor blocking activity was assessed by antagonism of (–)-NE-induced contractions of rat vas deferens (α_{1A}) or thoracic aorta (α_{1D}) and by antagonism of (–)-phenylephrine-induced contractions of rat spleen (α_{1B}). pA_2 values are means $\pm$ SEM of three different concentrations, each tested at least four times. ^c pK_B values ($\pm$ SEM) were calculated according to van Rossum. ^d The activity was determined by using a CellTiter-Glo luminescent cell viability assay (Promega). The data, expressed as percentage of control, are reported as IC_{50} values, which represents the concentration (μM) of compounds required for inhibition of 50% of cell growth. Each value is the mean of two independent experiments performed in duplicate, with the standard deviation ranging from 2.4 to 33.5%. ^e Data from ref 12. ^f Data from ref 24. ^g These data indicate that no antiproliferative activity was observed until at least the maximal tested dose (10 μM).

of doxazosin into a *cis*- and *trans*-decahydroquinoxaline nucleus to give **1** and **2**, respectively, is detrimental for the affinity at tissutal α_1 -adrenoceptors. However, the *cis* isomer **1** was more potent than the *trans* **2** at all the subtypes. This is in agreement with the reported higher affinity of cyclazosin relative to its *trans* isomer at the epididymal rat vas deferens α_1 -adrenoceptors.⁸ Notably, unlike **3**, **1** was significantly more potent at the α_{1B} subtype relative to α_{1A} and α_{1D} adrenoceptors (12- and 8-fold, respectively), whereas **2** was more potent at α_{1B} and α_{1D} subtypes with respect to the α_{1A} (18- and 10-fold, respectively).

In the present study, cyclazosin did not display any significant selectivity in functional experiments, in contrast to the results in binding assays.^{9,12} Furthermore, in binding assays, the affinity of **1–3** and cyclazosin at α_1 -adrenoceptor subtypes did not parallel the affinity observed in functional experiments as already found for a number of other prazosin-related compounds.²²

In Vitro Cytotoxic Activity. The antiproliferative effects of **1–3**, **1a**, and doxazosin were evaluated in vitro in PC-3, DU-145, and LNCaP human prostate cancer cells as well as in normal human dermal fibroblasts (NHDF) (Table 1).

Compounds **1** and **3** displayed antiproliferative activity in all tested cancer cells. In particular, **1** exhibited the highest effect, at submicromolar concentrations, whereas **3** was about 10-fold less potent than **1**. The *trans* isomer **2** was 17-fold less potent than **1** at LNCaP cells, and no effect was observed in the other cells up to the maximal tested concentration (10 μM). Compound **1** was 39–49-fold and 8–10-fold more potent on prostate malignant cells than doxazosin and **3**, respectively. Interestingly, **1a** was almost as active as **1**

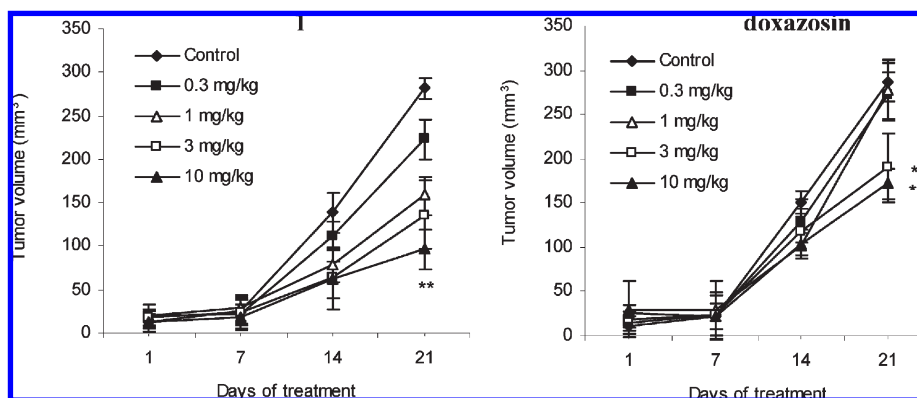


Figure 2. Effect of treatment with **1** and doxazosin on the growth of subcutaneous tumor implanted in male nude mice by human prostate PC-3 cancer cells. Each drug was administered orally and daily, for 21 consecutive days, at specific concentrations to five groups of mice (six animals/group, $n = 6$), under treatment and control. Data represent the mean $\pm$ SEM. Statistical analysis was done by analysis of variance (Anova) and Student t -test; **: significantly different from control, $p < 0.01$.

in PC-3 cells, suggesting that the stereochemistry, at least in these cells, may not have a relevant role in the antiproliferative activity. Concerning **2**, its activity was slightly higher than that of doxazosin in LNCaP cells (3-fold), but not comparable in the others. Like doxazosin, **1–3** displayed an antiproliferative effect at NHDF cells that was close to that shown in prostate cancer lines.

Apoptosis study. Different studies indicated that quinazoline-based α_1 -adrenoceptor antagonists, such as doxazosin, suppress prostate tumor growth via a plurality of mechanisms, including receptor-mediated apoptosis.⁷ Thus, given the structural similarity with doxazosin, the effect of **1–3** on apoptosis was evaluated in PC-3, DU-145, and LNCaP human prostate cancer cells and in NHDF cells through the evaluation of their ability in activation of effector caspases. The level of apoptosis was estimated by measuring in cells the activity of caspase-3 and caspase-7 that are considered the key effector of apoptosis in mammals.²³

Compound **1** was the most effective in caspase induction in cancer cells relative to **2**, **3**, and doxazosin (see Figure 1 of Supporting Information). This finding is in agreement with the higher antiproliferative effect observed for **1** (Table 1). Interestingly, **1**, unlike **2** and **3**, was, at the least, 4–7-fold less effective in inducing caspase activation and apoptosis in NHDF cells ($EC_{50} > 10 \mu M$) than in tumoral PC-3, DU-145, and LNCaP cells ($EC_{50} = 1.86, 2.60$, and $1.42 \mu M$, respectively). On the contrary, the effect of doxazosin at NHDF ($EC_{50} = 40.4 \mu M$) was similar to that on LNCaP cells but, at the least, 2.5-fold higher than in PC-3 and DU-145 cells ($EC_{50} > 100 \mu M$).

In Vivo Antitumor Activity. Given its interesting caspase induction ability and antiproliferative activity, **1** was chosen for further in vivo investigation in male nude mice bearing a subcutaneous tumor implanted in the left flank by injection of PC-3 cells. Figure 2 shows the course of tumor inhibition by **1** and doxazosin. A graphical representation of the different efficacy, at each tested concentration at the end of treatment, displayed by **1** and doxazosin, is reported in Figure 2 of Supporting Information.

Compound **1** inhibited the growth of the PC-3 induced tumor in a concentration-dependent fashion, which was significant at the minimal tested dose (21% decrease at 0.3 mg/kg vs control). In contrast, doxazosin gave a significant inhibition only at higher concentrations (3 and 10 mg/kg) (Figure 2 and Figure 2 of Supporting Information).

To confirm the in vitro observed apoptosis induced by **1** and doxazosin, this effect was also investigated in the removed subcutaneous tumor. At the same time, the tumor samples were employed to assess a possible antiangiogenic effect by **1** and doxazosin. The apoptotic effect of **1** and doxazosin was determined by a DNA fragmentation assay in which, through an electrophoretic analysis, a typical DNA ladder pattern was observed on tumor treated with drugs (see Figure 3 of Supporting Information). Again, **1** was more active than doxazosin because, approximately, in tumor treated with **1**, the characteristic pattern was observed at a concentration lower than in doxazosin-treated tumor (0.3 mg/kg vs 3 mg/kg).

The antiangiogenic activity of **1** and doxazosin was determined through immunohistochemical analysis by using the endothelial cell marker rat antimouse CD31 monoclonal antibody. Both drugs affected angiogenesis (see Figure 4 of Supporting Information). At a dose of 3 mg/kg, the drugs reduced blood vessel development. However, tumors treated with **1** were less vascularized than those treated with doxazosin and the effects of **1** on the tumors angiogenesis were also evident at dose of 0.3 and 1 mg/kg.

Conclusions

A few doxazosin analogues (**1–3** and **1a**) were investigated to assess their α_1 -adrenoceptor antagonism and antitumoral effects. Compound **1** displayed a better biological profile than doxazosin and hence a preferential position in potential therapeutic treatment. In particular, **1** was a potent and moderately selective α_{1B} -adrenoceptor antagonist, showing in vitro antiproliferative activity in PC-3, DU-145, and LNCaP human prostate cancer cells, at submicromolar concentrations, and also in vivo antitumor activity in mice PC-3-induced subcutaneous tumor. Although the results do not provide an explanation for the relationship between the α_1 -adrenoceptor antagonism and antitumor activity, compound **1**, bearing a *cis*-octahydroquinoxaline moiety, might be a useful lead compound for designing new compounds with improved biological profile.

Experimental Section

2-[4-(2,3-Dihydro-1,4-benzodioxin-2-ylcarbonyl)-*cis*-octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (1**).** A solution of **4** (1.10 g, 5.54 mmol) in dry CH_2Cl_2

(25 mL) was added dropwise, under nitrogen, to a stirred and cooled (0 °C) solution of **5** (4.20 g, 12.20 mmol) in dry CH₂Cl₂ (75 mL), whereupon the stirring was continued at room temperature for 24 h. Removal of the solvent gave a residue that was purified by silica column chromatography eluting with petroleum ether–ethyl acetate–methanol–14% ammonia (4:4:1:0.1). The obtained free base was transformed into the hydrochloride salt (see Supporting Information for analytical characterization).

(-)-2-[4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)-octahydroquinoxalin-1(2H)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (**1a**). It was obtained from **4** and **6**¹⁴ following a procedure similar to that described for **1** (see Supporting Information).

2-[4-(2,3-Dihydro-1,4-benzodioxin-2-ylcarbonyl)-trans-octahydroquinoxalin-1(2H)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride (**2**). It was obtained from **4** and **7** following the procedure described for **1** (see Supporting Information).

Acknowledgment. The present work was supported by grants from Fondazione Cassa di Risparmio della Provincia di Macerata (CARIMA), Macerata (Italy), and University of Camerino, Camerino (Italy).

Supporting Information Available: Analytical characterization of **1**, synthetic procedures and analytical characterization of **1a**, **2**, and **7**, elemental analyses, biological methods, in vitro caspase induction and in vivo antitumor efficacy and, apoptotic and antiangiogenic effects of **1** and doxazosin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Synthesis and α_1 -adrenoceptor antagonist activity of derivatives and isosters of the furan portion of (+)-cyclazosin

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Received 13 September 2006; revised 3 January 2007; accepted 17 January 2007

Available online 19 January 2007

Abstract— α_1 -Adrenoceptor selective antagonists are crucial in investigating the role and biological functions of α_1 -adrenoceptor subtypes. We synthesized and studied the α_1 -adrenoceptor blocking properties of new molecules structurally related to the α_{1B} -adrenoceptor selective antagonist (+)-cyclazosin, in an attempt to improve its receptor selectivity. In particular, we investigated the importance of substituents introduced at position 5 of the 2-furan moiety of (+)-cyclazosin and its replacement with classical isosteric rings. The 5-methylfuryl derivative (+)-**3**, [(+)-metacyclazosin], improved the pharmacological properties of the progenitor, displaying a competitive antagonism and an 11 fold increased selectivity for α_{1B} over α_{1A} , while maintaining a similar selectivity for the α_{1B} -adrenoceptor relative to the α_{1D} -adrenoceptor. Compound (+)-**3** may represent a useful tool for α_{1B} -adrenoceptor characterization in functional studies.

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1. Introduction

α_1 -Adrenoceptors are members of the G-protein coupled superfamily of receptors which play critical roles in the regulation of a variety of physiological processes. At present, three native subtypes, α_{1A} , α_{1B} and α_{1D} , have been pharmacologically detected,¹ and a fourth α_1 -adrenoceptor, α_{1L} , displaying low affinity for prazosin² and conformationally related to the α_{1A} -subtype,³ has been reported.

α_1 -Adrenoceptors are of therapeutic interest because of their important role in the control of blood pressure and in the contraction and growth of smooth and cardiac muscle.⁴ However, the functional role of specific α_1 -adrenoceptor subtypes is not completely defined because of the lack of ligands that are functionally selective for the α_1 -adrenoceptor subtypes. There is a particular need

for selective α_{1B} -adrenoceptor antagonists which, to date, have been discovered in very restricted number, in comparison to the selective α_{1A} - and α_{1D} -adrenoceptor ligands.

In fact, besides the alkylating agent chloroethylclonidine that was originally identified as selective α_{1B} -adrenoceptor antagonist,⁵ only a few reversible antagonists have been reported to be selective for the α_{1B} -adrenoceptor, including spiperone,⁶ risperidone,⁷ ($\pm$)-cyclazosin,⁸ L-765,314^{9,10} and AH11110A,¹¹ but their moderate selectivity, observed in radioligand binding assays, was not confirmed, with the exception of L-765,314, in functional experiments.¹²

Recently, we reported the quinazoline compound (+)-cyclazosin, (+)-2-[(4a*S*,8a*R*)-4-(2-furoyl)octahydroquinaxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(+)-**1**], which was characterized both for α_1 -adrenoceptor binding activity¹³ and chemical structure^{14,15} (Fig. 1). In binding assays, (+)-**1** displayed high affinity and selectivity for α_{1B} -adrenoceptor both in animal and human cloned α_1 -subtypes (pK_i α_{1B} = 9.16–9.87; selectivity ratios, α_{1B}/α_{1A} = 48–91 and

Keywords: α_1 -Adrenoceptor subtypes; α_1 -Adrenoceptor antagonists; α_{1B} -Adrenoceptor selective antagonists; (+)-Cyclazosin analogues.

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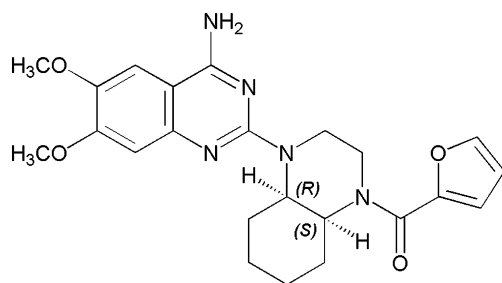


Figure 1. Structure of (+)-cyclazosin, (+)-2-[(4a*S*,8a*R*)-4-(2-furoyl)-octahydroquinoxalin-1 (2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine.

$\alpha_{1B}/\alpha_{1D} = 24\text{--}39$).¹³ Its α_{1B} -adrenoceptor selectivity was also confirmed in functional experiments performed in rat and rabbit tissues.¹⁶ It displayed competitive antagonism and higher potency at the α_{1B} -adrenoceptor ($pA_2 = 8.85$) than at α_{1A} ($pA_2 = 7.75$) and α_{1D} ($pA_2 = 7.27$) subtypes, with α_{1B}/α_{1A} - and α_{1B}/α_{1D} -selectivity ratio values of 13 and 38, respectively.

To improve the α_{1B} -adrenoceptor selectivity of (+)-1, we performed two kinds of structural modifications on its furan ring, while keeping the same structural configuration of carbon atoms 4a and 8a of the octahydroquinoxaline moiety: (i) introduction of selected substituents at position 5 of the furan ring, and (ii) replacement of the furan moiety with classical isosteric rings (Scheme 1). Thus, we report here the synthesis and the α_1 -adrenoceptor antagonist activity of the (+)-1 derivatives (+)-2 - (+)-5, bearing the bromo, methyl, methoxy or acetyl substituents at furan position 5,¹⁷ and the analogues (+)-6 - (+)-9, obtained by exchanging the furan moiety with a thiophene, pyrrole, pyridine or phenyl ring.

2. Chemistry

The two series of compounds (+)-2 - (+)-5 and (+)-6 - (+)-9, bearing the same stereochemical configuration of prototype (+)-1, were prepared by acylation of the

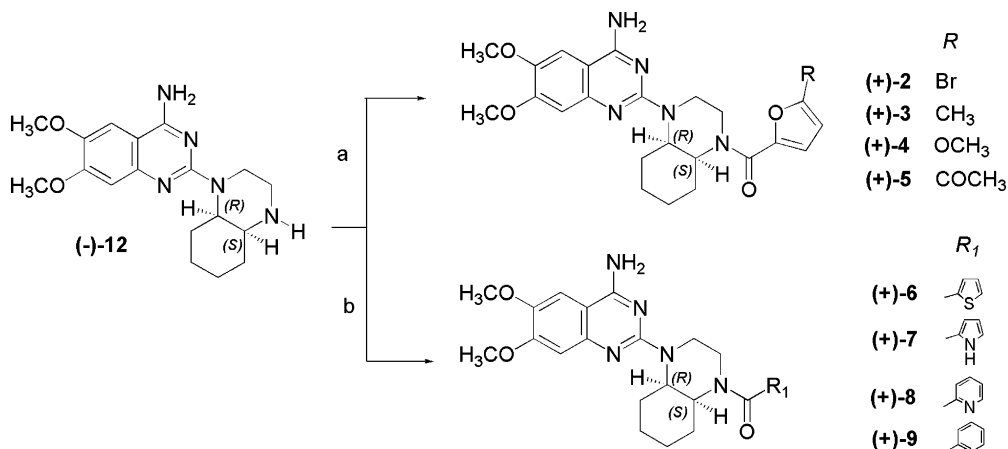
key intermediate (–)-12 with the appropriate 5-substituted-2-furoyl chloride or aryl chloride (Scheme 1).

Mono Cbz-protected (±)-10, synthesized by reaction between *cis*-decahydroquinoxaline¹⁸ and benzylchloroformate, was separated into the enantiomers (+)-10 and (–)-10 through fractional crystallization of the diastereomer salt mixture obtained by its treatment with (*S*)-(+)- or (*R*)-(–)-mandelic acid (Scheme 2).

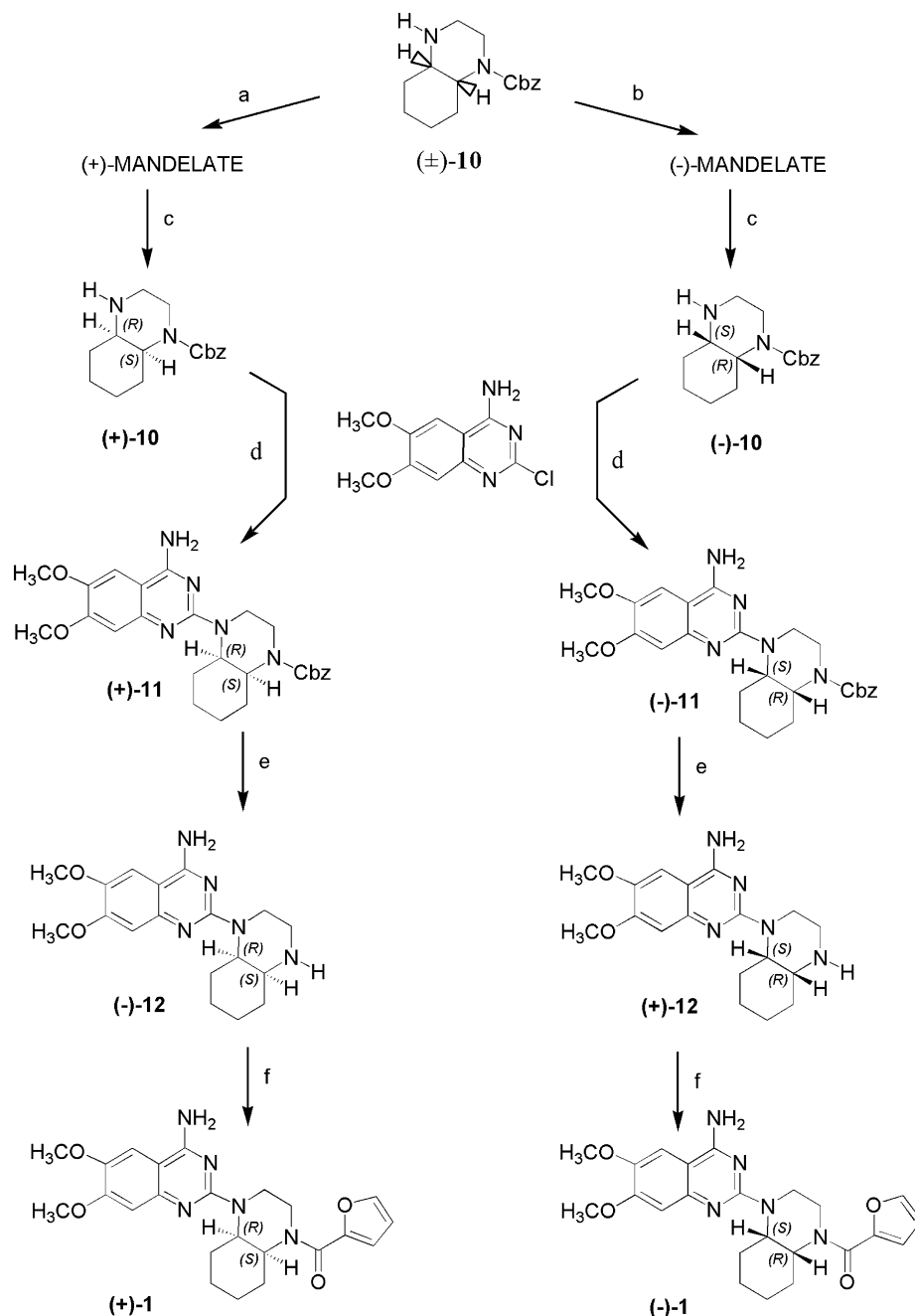
The configuration of (+)-10 and (–)-10 was deduced by synthesizing (+)-1 and (–)-1, whose absolute configuration is already known¹⁴ (Scheme 2). Thus, (+)-10 and (–)-10 were reacted with 2-chloro-6,7-dimethoxyquinazolin-4-amine to give (+)-11 and (–)-11, respectively. The subsequent removal of the Cbz protecting group by catalytic hydrogenation with cyclohexene and Pd/C in ethanol afforded the chiral amines (–)-12 and (+)-12, with inversion of optical rotation. Their acylation with 2-furoyl chloride gave two compounds that were identified by elemental analysis, ¹H NMR and optical rotatory parameters to be (+)-1 and (–)-1, respectively.

The enantiomeric purity of (+)-10 and (–)-10 was assessed by performing an HPLC analysis (HP Hypersil column, eluent mixture ethyl acetate/*n*-hexane) on the corresponding diastereomeric amides prepared by reaction with (*S*)-(–)-1-[(4-methylphenyl)sulfonyl]prolyl chloride,^{19,20} in comparison with the amide mixture obtained from (±)-10. Thus, the mixture obtained from (±)-10 showed a diagram with two peaks (retention time, 7.60 and 8.75 min), whereas the diastereomeric amides deriving from (+)-10 and (–)-10 displayed only one peak at retention time of 7.49 and 8.61 min, respectively (Fig. 2). The optical purity of (+)-10 and (–)-10 was found to be higher than 98%, since the analysis of an actual 98:2 mixture of these diastereomeric amides displayed, besides the principal peak, a significant and detectable small peak relative to the less abundant diastereomer.

The new chiral compounds (+)-2, (+)-3 and (+)-5 - (+)-9 were obtained as hydrochloride salts, purified by crystallization and characterized by elemental analysis, ¹H



Scheme 1. Reagents: (a) 5-substituted-2-furoyl chlorides, CH₂Cl₂, Et₃N; (b) R₁COCl, CH₂Cl₂, Et₃N.



Scheme 2. Reagents and conditions: (a) (*S*)-(+)-mandelic acid, MeOH; (b) (*R*)-(-)-mandelic acid, MeOH; (c) 2 N NaOH, H₂O/CHCl₃; (d) *i*-AmOH, reflux; (e) cyclohexene, Pd/C, MeOH/EtOH, reflux; (f) 2-furoyl chloride, Et₃N, CH₂Cl₂.

NMR, specific rotation and chromatographic parameters. Compound (+)-4 was isolated and characterized as the free base because of stability problem of the salt.

3. Pharmacology

Compounds (+)-2 - (+)-9 were studied in rat isolated tissues to assess their antagonist activity and selectivity at α_1 -adrenoceptor subtypes in comparison with the prototype (+)-1. α_{1A} - and α_{1D} -Adrenoceptor blocking activities were evaluated by antagonism of (-)-noradrenaline-induced contractions of prostatic vas deferens²¹ and aorta,²² respectively.

Stam et al.²³ reported a significantly lower affinity ($pK_B = 7.96$) for (+)-1 in α_{1B} -adrenoceptor rat spleen with respect to our results found in α_{1B} -adrenoceptor rabbit thoracic aorta ($pK_B = 8.85$),¹⁶ or in binding experiments on hamster cloned α_{1B} -adrenoceptors ($pK_i = 9.16$).¹³ For this reason, we decided to investigate the α_{1B} -adrenoceptor antagonism of (+)-1 and of its related compounds (+)-2 - (+)-9 in the rat spleen by using the Buckner procedure²⁴ with phenylephrine as agonist and an incubation period of 30 min (instead of 60 min as adopted by Stam et al.²³) because, in our experiments, the reproducibility of phenylephrine concentration-response curves was not observed when the curve was constructed after 45 and 60 min from the

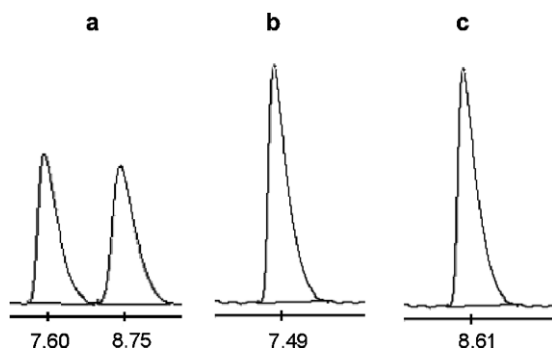


Figure 2. Analytical HPLC chromatograms of diastereomeric 1-[(4-methylphenyl)sulfonyl]prolyl amides obtained from racemic **10** (a) and from enantiomers (+)-**10** (b) and (–)-**10** (c). Chromatographic conditions: HP Hipersil column, 5 μ m (200 $\times$ 2.1 mm, i.d.); hexane/ethyl acetate (70:30, v/v) as eluting mixture at a flow rate of 1 mL/min; detection, UV at 254 nm. Retention times (min) are indicated at each peak.

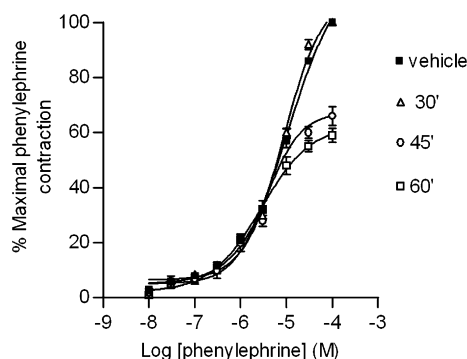


Figure 3. Time-dependent change of phenylephrine concentration–response curves in rat spleen, taken 30, 45, and 60 min after the curve used as control. Data are means $\pm$ SEM of six separate experiments.

one taken as control, owing to a 34–41% decrease of the maximal response to the agonist (Fig. 3).

The antagonist potency at α_1 -adrenoceptor subtypes was expressed as pA_2 values calculated by Schild plots at three different concentrations according to Arunlakshana and Schild.²⁵ However, when the slope of the Schild plot was significantly different from unity, the potency was expressed as pK_B values, according to van Rossum.²⁶ In this case, the pK_B value was calculated at the lowest antagonist concentration giving a significant rightward shift of the agonist concentration–response curve [$\log(\text{concentration ratio} - 1) \geq 0.5$].

The affinity profile of the new compounds was also evaluated in radioreceptor binding assays on human cloned α_1 -adrenoceptors in comparison with (+)-**1**. Competition experiments were performed using [^{3} H]prazosin to label α_1 -adrenoceptor binding sites on membranes of Chinese hamster ovary (CHO) cells expressing human α_{1a} -, α_{1b} - and α_{1d} -adrenoceptor subtypes.²⁷ Binding affinities were expressed as pK_i values derived using the Cheng–Prusoff equation.²⁸

Experimental data were subjected to statistical analysis by means of Student's *t*-test. A *p* value <0.05 was taken to indicate a statistically significant difference.

4. Results and discussion

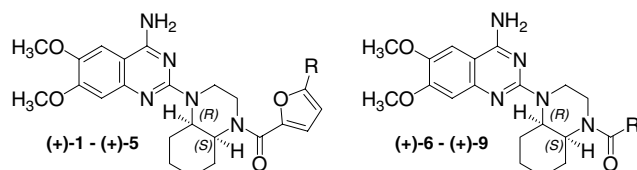
The pharmacological results of functional antagonism and radioligand binding assays are reported in Tables 1 and 2, respectively.

In functional assays, all the new compounds (+)-**2**–(+)-**9** were either competitive (mainly at α_{1D} and α_{1A} -adrenoceptors) or non-competitive antagonists (mainly at α_{1B} -adrenoceptor). In contrast with the competitive behaviour at rabbit aorta α_{1B} -adrenoceptor ($pA_2 = 8.85^{16}$), in the rat spleen α_{1B} -adrenoceptor (+)-**1** exhibited a non-competitive antagonism while displaying a similar potency ($pK_B = 8.86$). In this latter tissue, apart from (+)-**3** which competitively antagonized the phenylephrine-induced contractions in the 10–100 nM range, all new compounds were non-competitive antagonists, because the slope of the Schild plot was different from unity and the maximum response to the agonist was always reduced. Furthermore, it should be noted that compounds (+)-**2**–(+)-**5** displayed pK_B values different from those previously reported likely because an increased number of experiments were performed using higher concentrations of each compound.¹⁷

Both series of compounds revealed the same preferential antagonist activity for the α_{1B} -adrenoceptor over the α_{1A} - and α_{1D} -subtypes. The pK_B or pA_2 values of (+)-**2**–(+)-**5** at α_{1B} and α_{1D} -adrenoceptors ranged within 8.13–8.65 and 6.67–7.23, respectively, which are comparable with those displayed by (+)-**1** at the same adrenoceptor subtypes (8.86 and 7.27, respectively). These compounds displayed an α_{1B} -adrenoceptor selectivity relative to the α_{1D} -adrenoceptor similar to that of (+)-**1** (19–41 vs 39). In contrast, (+)-**3** and (+)-**4**, owing to a lower antagonism at the α_{1A} -adrenoceptor (6.48 and 6.85 vs 7.77), displayed an α_{1B}/α_{1A} selectivity ratio higher than that of (+)-**1** (148 and 45, respectively, vs 13).

The replacement of the furan ring of (+)-**1** with other isosteric aromatic moieties, affording the series (+)-**6**–(+)-**9**, did not modify the potency at the α_{1D} -adrenoceptor (7.12–7.76 vs 7.27). In addition, the activity was maintained also at the α_{1B} and α_{1A} -adrenoceptors with the exception of the slight decrease (5- to 6-fold) observed for the pyrrole analogue (+)-**7** at both subtypes and for the pyridine analogue (+)-**8** at the α_{1A} -adrenoceptor. Consequently, (+)-**8** was the most selective of this series with α_{1B}/α_{1D} and α_{1B}/α_{1A} selectivity ratios of 33 and 40, respectively.

In binding assays, the two series of compounds displayed higher affinity, although lower than that of prototype (+)-**1**, for the α_{1b} -adrenoceptor with respect to the other subtypes. Only the pyrrole analogue (+)-**7** showed similar affinity at α_{1b} - and α_{1d} -adrenoceptors. Moreover, these compounds displayed an affinity comparable to that of (+)-**1** at α_{1a} - and α_{1d} -adrenoceptor subtypes with the exception of (+)-**8** that was 8 fold less active than (+)-**1**

Table 1. Functional antagonist affinities, expressed as pA_2 or pK_B , of compounds (+)-**2**–(+)-**9**, and (+)-**1** as reference, at α_1 -adrenoceptor subtypes of isolated rat prostatic vas deferens (α_{1A}), spleen (α_{1B}) and thoracic aorta (α_{1D})


Compound	R/R ₁	pA_2^a or pK_B^b			Selectivity ratio ^c		
		α_{1A}	α_{1B}	α_{1D}	α_{1B}/α_{1A}	α_{1B}/α_{1D}	α_{1D}/α_{1A}
(+)- 1	Hydrogen	7.77 ± 0.03 ^a	8.86 ± 0.03 ^b	7.27 ± 0.07 ^a	13	39	0.3
(+)- 2	Bromo	7.53 ± 0.04 ^b	8.50 ± 0.02 ^b	7.23 ± 0.02 ^a	9	19	0.5
(+)- 3	Methyl	6.48 ± 0.12 ^a	8.65 ± 0.01 ^a	7.04 ± 0.03 ^a	148	41	4
(+)- 4	Methoxy	6.85 ± 0.02 ^a	8.50 ± 0.16 ^b	7.05 ± 0.02 ^a	45	28	2
(+)- 5	Acetyl	7.05 ± 0.04 ^b	8.13 ± 0.12 ^b	6.67 ± 0.09 ^a	12	29	0.4
(+)- 6	2-Thienyl	7.44 ± 0.07 ^b	8.78 ± 0.09 ^b	7.45 ± 0.06 ^a	22	21	1
(+)- 7	2-Pyrrolyl	7.06 ± 0.06 ^b	8.05 ± 0.02 ^b	7.33 ± 0.02 ^b	10	5	2
(+)- 8	2-Pyridyl	7.04 ± 0.13 ^b	8.64 ± 0.14 ^b	7.12 ± 0.02 ^b	40	33	1
(+)- 9	Phenyl	7.51 ± 0.01 ^a	8.83 ± 0.07 ^b	7.76 ± 0.04 ^b	21	12	2

^a pA_2 values, expressed as means ± SEM of three different concentrations, each tested at least four times.^b pK_B values (±SEM) calculated according to van Rossum.^c Calculated by the antilog of the difference between pA_2 or pK_B values at different α_1 -adrenoceptor subtypes.**Table 2.** Binding affinity constants, expressed as pK_i , of compounds (+)-**2**–(+)-**9**, and (+)-**1** as reference, at cloned human α_1 -adrenoceptor subtypes expressed in CHO cells

Compound	pK_i^a			Selectivity ratio ^b		
	α_{1a}	α_{1b}	α_{1d}	α_{1b}/α_{1a}	α_{1b}/α_{1d}	α_{1d}/α_{1a}
(+)- 1	7.91	9.87	8.49	91	24	4
(+)- 2	8.46	9.51	8.81	11	5	2
(+)- 3	7.63	9.06	8.32	27	6	5
(+)- 4	7.56	9.06	8.44	32	4	8
(+)- 5	7.59	8.86	8.13	19	5	4
(+)- 6	8.37	9.62	8.63	18	10	2
(+)- 7	7.98	8.50	8.82	3	0.5	7
(+)- 8	7.31	8.23	7.57	8	5	2
(+)- 9	8.38	9.31	8.49	8.5	7	1

^a Equilibrium dissociation constants (K_i) were calculated from IC_{50} values using the Cheng–Prusoff equation. The affinity estimates, derived from displacement of [³H]prazosin binding from α_1 -adrenoceptors, were from two to three experiments performed in triplicate, which agreed within ±20%.^b Calculated by the antilog of the difference between pK_i values at different α_1 -adrenoceptor subtypes.

at α_{1d} -adrenoceptors. Consequently, the new compounds maintained, although to a lesser extent, the α_{1b} -adrenoceptor selectivity of (+)-**1** with the exception of (+)-**7** that was 7-fold selective for the α_{1d} -adrenoceptor over the α_{1a} subtype. The most selective compounds of the series were (+)-**3** and (+)-**4** with an α_{1b}/α_{1a} selectivity ratio of 27 and 32, respectively, and the thienyl analogue (+)-**6** with α_{1b}/α_{1a} and α_{1b}/α_{1d} selectivity ratios of 18 and 10.

A comparison between binding and functional data clearly shows that the results are not comparable because pK_B or pA_2 values are lower than the pK_i values at all α_1 -adrenoceptor subtypes. This discrepancy was already observed for other α_1 -adrenoceptor antagonists, including prazosin-related compounds, and different hypothe-

ses were taken into account for a possible explanation: diffusion-related temporal inequilibrium,²⁹ inverse agonism and heterodimer formation.^{30–32} Thus, the discrepancy observed in the present work may be the consequence of different bioavailabilities of compounds at the receptor level or of species differences in affinity.³²

5. Conclusion

The results of the present investigation indicate that, besides the unsubstituted and 5-substituted furans, other aromatic rings, like thiophene, pyridine and phenyl, confer high potency towards the α_{1b} -subtype. Among the studied compounds, the 5-methyl furyl derivative (+)-**3**, [(+)-metcyclazosin], displayed the most interesting

pharmacological profile. In contrast to the progenitor and other analogues, it displayed a competitive antagonism at all three α_1 -adrenoceptor subtypes and improved selectivity for the α_{1B} -adrenoceptor. In particular, (+)-**3** showed 11-fold higher α_{1B}/α_{1A} selectivity ratio (148) relative to (+)-**1** (13), while keeping similar selectivity for the α_{1B} -adrenoceptor over the α_{1D} -adrenoceptor subtype (ratio 41 vs 39). Since very few selective α_{1B} -adrenoceptor ligands are available, (+)-**3** may represent a useful tool for α_{1B} -adrenoceptor characterization in functional studies.

6. Experimental

6.1. Chemistry

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR and NMR spectra were recorded on Perkin-Elmer 297 and Varian VXR 300 instruments, respectively. The IR spectra, not included, were consistent with all the assigned structures. The elemental analyses of compounds, performed on a Fisons instrument mod. EA1108CHNS-O, agreed with the calculated values within the range $\pm 0.4\%$. Electron impact ionization (EI) mass spectra were obtained with a Hewlett-Packard instrument, consisting of model 5890 A for the separation section and model 5971 A for the mass section. Electrospray (ESI) mass spectra were recorded on a Hewlett-Packard series 1100 MSD spectrometer. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. Analytical HPLC was performed on a Hewlett-Packard 1090 apparatus, series II, with UV detector, equipped with a HP Hypersil column, 5 μm (200×2.1 mm, i.d.). Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040–0.063 mm, Merck) by flash chromatography. R_f values were determined with silica gel TLC plates (Kieselgel 60 F₂₅₄, layer thickness 0.25 mm, Merck). The composition and volumetric ratio of eluting mixtures were: A, petroleum ether–ethyl acetate–methanol–28% ammonia (8:6:2:0.2); B, petroleum ether–ethyl acetate–methanol–28% ammonia (8:6:2:0.1); C, petroleum ether–ethyl acetate–methanol–28% ammonia (9:6:1.5:0.1); D, petroleum ether–ethyl acetate–methanol–28% ammonia (8:6:2:0.05); E, petroleum ether–ethyl acetate–methanol–7% ammonia (6:4:2:0.2); F, cyclohexane–ethyl acetate–methanol–14% ammonia (6:4:1.5:0.1); G, ethyl acetate–cyclohexane–chloroform–methanol (7:3:2:1); H, ethyl acetate–cyclohexane (5:5). Petroleum ether refers to the fraction with a boiling point of 40–60 °C. The term ‘dried’ refers to the use of anhydrous sodium sulfate. Compounds were named following IUPAC rules as applied by ACD/Name software, version 7.0 (Advanced Chemistry Development, Inc., Toronto, Canada). Solid compounds were purified by crystallization using the following solvents or mixture of solvents: MeOH, (+)-**2**, (+)-**6**, (+)-**7**; EtOH, (+)-**5**, (+)-**9**, (–)-**11**; *i*-PrOH, (+)-**3**, (+)-**11**; MeOH/EtOH, (+)-**1**, (–)-**1**; MeOH/*i*-PrOH, (+)-**12**, (–)-**12**. Yields are of purified products and were not optimized. Chemicals and reagents were purchased from Sigma–Aldrich Srl (Milano, Italy) or Lancaster

research chemicals (Chiminord, Srl, Cusano Milanino, Milano, Italy).

6.1.1. ($\pm$)-Benzyl *cis*-octahydroquinoxaline-1(2*H*)-carboxylate [($\pm$)-10**].** A solution of methanesulfonic acid (10 g, 104 mmol) in water (10 mL) was added to a mixture of *cis*-decahydroquinoxaline (8.21 g, 5.6 mmol), H₂O (12 mL) and EtOH (35 mL). Next, a solution of benzyl chloroformate (8.50 g, 49.8 mmol) in 1,2-dimethoxyethane (18 mL) and a 50% water solution of potassium acetate (6 mL) were alternatively added dropwise to the solution. The mixture was heated at 60 °C for 2 h and then evaporated under vacuum. The residue was treated with water and the resulting precipitate was removed by filtration. The solution was basified with 2 N NaOH and extracted with CHCl₃. The removal of dried solvent gave a crude residue that was purified by column chromatography eluting with mixture E to give ($\pm$)-**10** as an oily product: 10.5 g (68%); R_f = 0.28 (mixture F), MS (EI) m/z = 274 [M]⁺, ¹H NMR (CDCl₃): δ 1.22–2.21 (m, 9H, H_{5–8} octahydroquinoxaline and NH, exchangeable with D₂O), 2.68–3.20 (m, 4H, H_{2–3} octahydroquinoxaline), 3.78–4.15 (m, 2H, H_{4a} and H_{8a} octahydroquinoxaline), 5.18 (br s, 2H, OCH₂), 7.24–7.41 (m, 5H, aromatics).

6.1.1.1. Optical resolution of ($\pm$)-benzyl *cis*-octahydroquinoxaline-1(2*H*)-carboxylate [($\pm$)-10**].** A solution of racemic **10** (22.8 g, 83 mmol) in MeOH (100 mL) was treated with a solution of (*S*)-(+)-mandelic acid (12.6 g, 83 mmol) in MeOH (100 mL). Removal of the solvent gave a residue that was crystallized seven times from ethyl acetate, affording the less soluble (+)-mandelate salt: 5.5 g; mp 140–143 °C; $[\alpha]_D^{20}$ +61.6 (*c* 1, MeOH); ¹H NMR (DMSO-*d*₆): δ 1.15–2.08 (m, 8H, H_{5–8} octahydroquinoxaline), 2.61–3.15 (m, 5H, H_{2–3} octahydroquinoxaline and OH, exchangeable with D₂O), 3.70–4.00 (m, 2H, H_{4a} and H_{8a} octahydroquinoxaline), 4.88 (s, 1H, CH mandelic acid), 5.10 (br s, 2H, OCH₂), 6.60 (br signal, 2H, NH₂⁺, exchangeable with D₂O), 7.18–7.41 (m, 10H, aromatics). Anal. Calcd for C₂₄H₃₀N₂O₅: C, 67.59; H, 7.09; N, 6.57. Found: C, 67.44; H, 7.45; N, 6.63.

Following a similar procedure, the other diastereomer mandelate was obtained from racemic **10** (8 g, 29 mmol) and an equimolar amount of (*R*)-(–)-mandelic acid: 2.7 g; mp 140–142 °C; $[\alpha]_D^{20}$ –61.1 (*c* 1, MeOH); ¹H NMR (DMSO-*d*₆): δ 1.12–2.10 (m, 8H, H_{5–8} octahydroquinoxaline), 2.59–3.15 (m, 4H, H_{2–3} octahydroquinoxaline), 3.43 (br signal, 1H, OH, exchangeable with D₂O), 3.65–4.00 (m, 2H, H_{4a} and H_{8a} octahydroquinoxaline), 4.90 (s, 1H, CH mandelic acid), 5.12 (br s, 2H, OCH₂), 6.66 (br signal, 2H, NH₂⁺, exchangeable with D₂O), 7.18–7.46 (m, 10H, aromatics). Anal. Calcd for C₂₄H₃₀N₂O₅: C, 67.59; H, 7.09; N, 6.57. Found: C, 67.48; H, 7.39; N, 6.61.

6.1.1.2. (+)-Benzyl (4*aR*,8*aS*)-octahydroquinoxaline-1(2*H*)-carboxylate [(+)-10**].** The (+)-mandelate salt (5.30 g) was dissolved in H₂O and then displaced in basic medium by addition of 2 N NaOH at low temperature (0 °C). The free base was extracted with CHCl₃, the solvent dried and then evaporated to give 3.5 g of (+)-**10** as an oil: $[\alpha]_D^{20}$ +35.8 (*c* 0.5, MeOH); R_f = 0.28 (mixture

F); MS (EI) $m/z = 274$ $[M]^+$; ^1H NMR (CDCl_3): δ 1.22–2.21 (m, 9H, H_{5-8} octahydroquinoxaline, NH exchangeable with D_2O), 2.68–3.20 (m, 4H, H_{2-3} octahydroquinoxaline), 3.78–4.15 (m, 2H, H_{4a} and H_{8a} octahydroquinoxaline), 5.18 (br s, 2H, OCH_2), 7.24–7.41 (m, 5H, aromatics).

6.1.1.3. (–)-Benzyl (4a*S*,8a*R*)-octahydroquinoxaline-1(2*H*)-carboxylate [(–)-10]. The (–)-mandelate salt (2.28 g) was dissolved in H_2O and then displaced in basic medium by addition of 2 N NaOH at low temperature (0 °C). The free base was extracted with CHCl_3 that was dried and then evaporated to give 1.27 g of (–)-10 as an oil: $[\alpha]_{\text{D}}^{20} -36.0$ (c 0.5, MeOH); $R_f = 0.28$ (mixture F); MS (EI) $m/z = 274$ $[M]^+$; ^1H NMR (CDCl_3): δ 1.20–2.20 (m, 9H, H_{5-8} octahydroquinoxaline, NH exchangeable with D_2O), 2.66–3.18 (m, 4H, H_{2-3} octahydroquinoxaline), 3.75–4.15 (m, 2H, H_{4a} and H_{8a} octahydroquinoxaline), 5.15 (br s, 2H, OCH_2), 7.21–7.40 (m, 5H, aromatics).

6.1.1.4. Determination of the optical purity of (+)-10 and (–)-10. A solution of (*S*)-(–)-1-[(4-methylphenyl)sulfonyl]prolyl chloride³³ (0.31 g, 1.09 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise and under nitrogen to a cooled (0 °C) and stirred solution of racemic 10 (0.2 g, 0.73 mmol) and Et_3N (0.15 mL) dissolved in CH_2Cl_2 (12 mL). Then the mixture was stirred at room temperature for 3 h, the solvent evaporated and the crude residue purified by column chromatography (mixture H) to give an oily mixture of the two diastereomers of benzyl 4-{1-[(4-methylphenyl)sulfonyl]prolyl}-*cis*-octahydroquinoxaline-1(2*H*)-carboxylate: 0.30 g; MS (ESI) $m/z = 526$ $[(M+H)^+]$; ^1H NMR (CDCl_3): δ 1.25–2.60 (m, 15H, H_{5-8} octahydroquinoxaline, H_{3-4} proline and CH_3), 3.25–4.46 (m, 8H, H_{2-3} , H_{4a} and H_{8a} octahydroquinoxaline, H_5 proline), 4.62–4.80 (m, 1H, H_2 proline), 5.18 (s, 2H, OCH_2), 7.22–7.51 (m, 7H, aromatics), 7.70–7.83 (m, 2H, aromatics).

The two diastereomeric amides were synthesized as vitreous compounds by reaction of (*S*)-(–)-1-[(4-methylphenyl)sulfonyl]prolyl chloride alternatively with (+)-10 and (–)-10.

Amide from (+)-10: MS (ESI) $m/z = 526$ $[(M+H)^+]$; ^1H NMR (CDCl_3): δ 1.25–2.60 (m, 15H, H_{5-8} octahydroquinoxaline, H_{3-4} proline and CH_3), 3.23–4.15 (m, 7H, H_{2-3} , H_{4a} or H_{8a} octahydroquinoxaline and H_5 proline), 4.25–4.42 (m, 1H, H_{8a} or H_{4a}), 4.62–4.72 (m, 1H, H_2 proline), 5.18 (s, 2H, OCH_2), 7.20–7.43 (m, 7H, aromatics), 7.72–7.78 (m, 2H, aromatics).

Amide from (–)-10: MS (ESI) $m/z = 526$ $[(M+H)^+]$; ^1H NMR (CDCl_3): δ 1.25–2.60 (m, 15H, H_{5-8} octahydroquinoxaline, H_{3-4} proline and CH_3), 3.27–4.46 (m, 8H, H_{2-3} , H_{4a} and H_{8a} octahydroquinoxaline and H_5 proline), 4.62–4.78 (m, 1H, H_2 proline), 5.18 (s, 2H, OCH_2), 7.22–7.43 (m, 7H, aromatics), 7.71–7.82 (m, 2H, aromatics).

The HPLC analysis of the two single diastereomers, in comparison with a diastereomeric mixture of them,

was performed on a HP Hipersil column, 5 μm (200 $\times$ 2.1 mm, i.d.), with hexane/ethyl acetate (70:30, v/v) as eluting mixture at a flow rate of 1 mL/min. Under these conditions the injected samples of mixtures (3 μL , c 2.5 mg/mL), detectable by absorbance at 254 nm, produced two peaks with retention time of 7.60 and 8.75 min, whereas the two diastereomers obtained from (+)-10 and (–)-10 displayed single peaks at retention time of 7.49 and 8.61 min, respectively.

6.1.2. (+)-Benzyl (4a*R*,8a*S*)-4-(4-amino-6,7-dimethoxyquinazolin-2-yl)octahydroquinoxaline-1(2*H*)-carboxylate hydrochloride [(+)-11]. A mixture of 2-chloro-6,7-dimethoxyquinazolin-4-amine (1.27 g, 5.31 mmol), (+)-10 (1.60 g, 5.84 mmol) and Et_3N (2.25 mL, 15.93 mmol) in *i*-AmOH (40 mL) was stirred under reflux for seven days. The solvent was removed in vacuum, then the crude residue was triturated with cold 2 N NaOH and the precipitate collected. Purification by column chromatography, eluting with mixture G, afforded a solid that was transformed into (+)-11 as hydrochloride salt: 1.3 g (48%); mp 243–245 °C; $R_f = 0.37$ (mixture F); $[\alpha]_{\text{D}}^{20} +14.9$ (c 1, MeOH); ^1H NMR ($\text{DMSO}-d_6$): δ 1.25–1.98 (m, 7H, H_{5-8} octahydroquinoxaline), 2.28–2.47 (m, 1H, H_{5-8} octahydroquinoxaline), 3.63–4.28 (m, 11H, OCH_3 , H_{2-3} and H_{4a} octahydroquinoxaline), 4.60–4.87 (m, 1H, H_{8a} octahydroquinoxaline), 5.05–5.24 (m, 2H, OCH_2), 7.21–7.46 (m, 5H, aromatics), 7.65 (s, 1H, aromatics), 7.78 (s, 1H, aromatics), 8.68 (br s, 1H, NH, exchangeable with D_2O), 8.92 (br s, 1H, NH, exchangeable with D_2O), 12.10 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{26}\text{H}_{31}\text{N}_5\text{O}_4\cdot\text{HCl}\cdot 0.75\text{H}_2\text{O}\cdot \text{C}_3\text{H}_8\text{O}$: C, 59.28; H, 7.12; N, 11.92. Found: C, 59.42; H, 7.39; N, 12.30.

6.1.3. (–)-Benzyl (4a*S*,8a*R*)-4-(4-amino-6,7-dimethoxyquinazolin-2-yl)octahydroquinoxaline-1(2*H*)-carboxylate hydrochloride [(–)-11]. The title compound was synthesized from 2-chloro-6,7-dimethoxyquinazolin-4-amine (0.99 g, 4.2 mmol) and (–)-10 (1.27 g, 4.6 mmol) following the procedure described for (+)-11: 1.5 g (71%); mp 242–244 °C; $R_f = 0.37$ (mixture F); $[\alpha]_{\text{D}}^{20} -15.3$ (c 1, MeOH); ^1H NMR ($\text{DMSO}-d_6$): δ 1.23–2.00 (m, 7H, H_{5-8} octahydroquinoxaline), 2.28–2.47 (m, 1H, H_{5-8} octahydroquinoxaline), 3.70–4.20 (m, 11H, OCH_3 , H_{2-3} and H_{4a} octahydroquinoxaline), 4.62–4.80 (m, 1H, H_{8a} octahydroquinoxaline), 5.03–5.20 (m, 2H, OCH_2), 7.25–7.40 (m, 5H, aromatics), 7.63 (s, 1H, aromatics), 7.75 (s, 1H, aromatics), 8.62 (br s, 1H, NH, exchangeable with D_2O), 8.91 (br s, 1H, NH, exchangeable with D_2O), 12.02 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{26}\text{H}_{31}\text{N}_5\text{O}_4\cdot\text{HCl}\cdot\text{H}_2\text{O}\cdot 0.25\text{C}_2\text{H}_6\text{O}$: C, 58.56; H, 6.58; N, 12.88. Found: C, 58.58; H, 6.40; N, 12.90.

6.1.4. (–)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine dihydrochloride [(–)-12]. Cyclohexene (42 mL) and 10% Pd/C (0.56 g) were consecutively added to a solution of (+)-11 (1.2 g, 2.34 mmol) in MeOH (10 mL) and EtOH (30 mL). The mixture was stirred while heating at 80 °C for 6 h. After cooling, the catalyst was filtered off and the solvent evaporated to dryness to give a residue that was transformed into (–)-12 as the hydrochloride salt: 0.79 g

(81%); mp 287–290 °C; R_f = 0.24 (mixture A); $[\alpha]_D^{20}$ –60.1 (*c* 1, MeOH); ^1H NMR (DMSO- d_6): δ 1.35–2.40 (m, 8H, H_{5-8} octahydroquinoxaline), 2.97–3.65 (m, 4H, H_{2-3} octahydroquinoxaline), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.62–4.83 (m, 1H, H_{4a} octahydroquinoxaline), 4.88–5.20 (m, 1H, H_{8a} octahydroquinoxaline), 7.80 (s, 2H, aromatics), 8.74 (br s, 1H, NH, exchangeable with D_2O), 9.07 (br s, 1H, NH, exchangeable with D_2O), 9.60 (br s, 1H, NH exchangeable with D_2O), 10.05 (br s, 1H, NH exchangeable with D_2O), 12.82 (br s, 1H, NH exchangeable with D_2O). Anal. Calcd for $\text{C}_{18}\text{H}_{25}\text{N}_5\text{O}_2 \cdot 2\text{HCl} \cdot 1.5\text{H}_2\text{O}$: C, 48.76; H, 6.82; N, 15.80. Found: C, 48.99; H, 6.76; N, 15.85.

6.1.5. (+)-6,7-Dimethoxy-2-[(4a*R*,8a*S*)-octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine dihydrochloride [(+)-12]. Obtained from (–)-11 (1.73 g, 3.82 mmol) following the procedure described for (–)-12: 0.72 g (55%); mp 290–292 °C; R_f = 0.24 (mixture A); $[\alpha]_D^{20}$ +59.6 (*c* 1, MeOH); ^1H NMR (DMSO- d_6): δ 1.35–2.40 (m, 8H, H_{5-8} octahydroquinoxaline), 3.00–3.68 (m, 4H, H_{2-3} octahydroquinoxaline), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.62–4.83 (m, 1H, H_{4a} octahydroquinoxaline), 4.90–5.20 (m, 1H, H_{8a} octahydroquinoxaline), 7.80 (s, 2H, aromatics), 8.75 (br s, 1H, NH, exchangeable with D_2O), 9.00 (br s, 1H, NH, exchangeable with D_2O), 9.62 (br s, 1H, NH, exchangeable with D_2O), 10.00 (br s, 1H, NH, exchangeable with D_2O), 12.82 (br s, 1H, NH exchangeable with D_2O). Anal. Calcd for $\text{C}_{18}\text{H}_{25}\text{N}_5\text{O}_2 \cdot 2\text{HCl} \cdot 1\text{H}_2\text{O}$: C, 49.77; H, 6.73; N, 16.12. Found: C, 49.73; H, 6.88; N, 16.06.

6.1.6. (+)-2-[(4a*S*,8a*R*)-4-(2-Furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(+)-1]. A solution of 2-furoyl chloride (0.034 g, 0.26 mmol) in dry CH_2Cl_2 (2.5 mL) was added dropwise to a cooled (0 °C) and stirred solution of (–)-12 free base (0.060 g, 0.17 mmol) and Et_3N (0.02 mL) in dry CH_2Cl_2 (5 mL). Then the mixture was stirred for 3 h at room temperature, the solvent removed and the residue purified by column chromatography eluting with mixture C. The product was transformed into (+)-1 as hydrochloride salt: 0.022 g; mp 263–264 °C; R_f = 0.43 (mixture A); $[\alpha]_D^{20}$ +74.1 (*c* 1, MeOH); ^1H NMR (DMSO- d_6): δ 1.25–2.45 (m, 8H, H_{5-8} octahydroquinoxaline), 3.70–4.30 (m, 10H, H_{2-3} octahydroquinoxaline, OCH_3), 4.37–4.48 (m, 1H, H_{8a} octahydroquinoxaline), 4.58–4.76 (m, 1H, H_{4a} octahydroquinoxaline), 6.72 (m, 1H, H_4 furan), 7.14 (m, 1H, H_3 furan), 7.54 (s, 1H, aromatics), 7.76 (s, 1H, aromatics), 7.90 (m, 1H, H_5 furan), 8.62 (br s, 1H, NH, exchangeable with D_2O), 8.88 (br s, 1H, NH, exchangeable with D_2O), 11.90 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{23}\text{H}_{27}\text{N}_5\text{O}_4 \cdot \text{HCl} \cdot 1.5\text{H}_2\text{O}$: C, 55.14; H, 6.24; N, 13.98. Found: C, 55.29; H, 6.56; N, 14.16.

6.1.7. (–)-2-[(4a*R*,8a*S*)-4-(2-Furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(–)-1]. Obtained from (+)-12 free base (0.072 g, 0.21 mmol) following the procedure described for (+)-1: 0.018 g; mp 263–265 °C; R_f = 0.43 (mixture A); $[\alpha]_D^{20}$ –73.9 (*c* 1, MeOH); ^1H NMR (DMSO- d_6): δ 1.25–2.45 (m, 8H, H_{5-8} octahydroquinoxaline), 3.70–4.30 (m, 10H,

H_{2-3} octahydroquinoxaline, OCH_3), 4.37–4.50 (m, 1H, H_{8a} octahydroquinoxaline), 4.52–4.76 (m, 1H, H_{4a} octahydroquinoxaline), 6.72 (m, 1H, H_4 furan), 7.14 (m, 1H, H_3 furan), 7.56 (s, 1H, aromatics), 7.76 (s, 1H, aromatics), 7.90 (m, 1H, H_5 furan), 8.64 (br s, 1H, NH, exchangeable with D_2O), 8.90 (br s, 1H, NH, exchangeable with D_2O), 11.90 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{23}\text{H}_{27}\text{N}_5\text{O}_4 \cdot \text{HCl} \cdot 1.5\text{H}_2\text{O}$: C, 55.14; H, 6.24; N, 13.98. Found: C, 55.40; H, 6.12; N, 14.27.

6.1.8. General procedure for the synthesis of [(+)-2 - (+)-9]. A solution of proper acyl chloride (1.05 mmol) in dry CH_2Cl_2 (5 mL) was added dropwise to a stirred and cooled (0 °C) solution of (–)-12 free base (1 mmol) and Et_3N (0.15 mL, 1.5 mmol) dissolved in dry CH_2Cl_2 (10 mL). Then the mixture was stirred at room temperature for 3 h, the solvent evaporated and the residue purified by column chromatography. The products, eluted as free bases, were transformed into the corresponding hydrochloride salts and crystallized. However, (+)-4 was isolated and characterized as free base.

6.1.8.1. (+)-2-[(4a*S*,8a*R*)-4-(5-Bromo-2-furoyl)octahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(+)-2]. Obtained from 5-bromo-2-furoyl chloride³⁴ (0.16 g, 0.77 mmol) and (–)-12 free base (0.25 g, 0.73 mmol): 0.08 g (21%); mp 270–272 °C; R_f = 0.56 (eluting mixture B); $[\alpha]_D^{20}$ +90.8 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.20–2.10 (m, 7H, H_{5-8} octahydroquinoxaline), 2.35–2.42 (m, 1H, H_{5-8} octahydroquinoxaline), 3.78–4.32 (m, 10H, H_{2-3} octahydroquinoxaline, OCH_3), 4.38–4.50 (m, 1H, H_{8a} octahydroquinoxaline), 4.57–4.80 (m, 1H, H_{4a} octahydroquinoxaline), 6.82 (m, 1H, H_4 furan), 7.21 (m, 1H, H_3 furan), 7.55 (s, 1H, aromatics), 7.76 (s, 1H, aromatics), 8.65 (br s, 1H, NH, exchangeable with D_2O), 8.90 (br s, 1H, NH, exchangeable with D_2O), 12.00 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{23}\text{H}_{26}\text{BrN}_5\text{O}_4 \cdot \text{HCl} \cdot 0.25\text{H}_2\text{O}$: C, 49.56; H, 4.97; N, 12.57. Found: C, 49.41; H, 5.15; N, 12.41.

6.1.8.2. (+)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-4-(5-methyl-2-furoyl)octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine hydrochloride [(+)-3]. Obtained from 5-methyl-2-furoyl chloride³⁴ (0.11 g, 0.76 mmol) and (–)-12 free base (0.25 g, 0.73 mmol): 0.075 g (20%); mp 273–275 °C; R_f = 0.45 (eluting mixture B); $[\alpha]_D^{20}$ +70.2 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.20–2.10 (m, 7H, H_{5-8} octahydroquinoxaline), 2.20–2.45 (m, 4H, H_{5-8} octahydroquinoxaline and CH_3), 3.73–4.32 (m, 10H, H_{2-3} octahydroquinoxaline, OCH_3), 4.38–4.50 (m, 1H, H_{8a} octahydroquinoxaline), 4.57–4.73 (m, 1H, H_{4a} octahydroquinoxaline), 6.30 (m, 1H, H_4 furan), 7.00 (m, 1H, H_3 furan), 7.58 (s, 1H, aromatics), 7.78 (s, 1H, aromatics), 8.68 (br s, 1H, NH, exchangeable with D_2O), 8.90 (br s, 1H, NH, exchangeable with D_2O), 11.98 (br s, 1H, NH, exchangeable with D_2O). Anal. Calcd for $\text{C}_{24}\text{H}_{29}\text{N}_5\text{O}_4 \cdot \text{HCl} \cdot 3.5\text{H}_2\text{O} \cdot 0.25\text{C}_3\text{H}_8\text{O}$: C, 52.51; H, 6.94; N, 12.37. Found: C, 52.36; H, 6.60; N, 12.03.

6.1.8.3. (+)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-4-(5-methoxy-2-furoyl)octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine [(+)-4]. Obtained from 5-methoxy-2-furoyl chloride³⁵

(0.12 g, 0.76 mmol) and (–)-**12** free base (0.25 g, 0.73 mmol). Because of stability problem the product was isolated and characterized as free base: 0.05 g (12%); mp 139–141 °C; R_f = 0.27 (eluting mixture B); $[\alpha]_D^{20}$ +13.3 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.21–2.18 (m, 7H, H_{5–8} octahydroquinoxaline), 2.20–2.42 (m, 1H, H_{5–8} octahydroquinoxaline), 3.70–4.18 (m, 13H, H_{2–3} octahydroquinoxaline, OCH₃), 4.22–4.38 (m, 1H, H_{8a} octahydroquinoxaline), 4.42–4.60 (m, 1H, H_{4a} octahydroquinoxaline), 5.60 (m, 1H, H₄ furan), 6.78 (br s, 1H, aromatics), 7.05 (m, 1H, H₃ furan), 7.20 (br s, 2H, NH, exchangeable with D₂O), 7.42 (br s, 1H, aromatics). Anal. Calcd for C₂₄H₂₉N₅O₅·1.5H₂O·0.25C₆H₁₄: C, 59.35; H, 6.93; N, 13.57. Found: C, 59.43; H, 6.75; N, 13.43.

6.1.8.4. (+)-1-(5-[(4a*R*,8a*S*)-4-(4-Amino-6,7-dimethoxyquinazolin-2-yl)octahydroquinoxalin-1(2*H*)-yl]carbonyl]-2-furyl)ethanone hydrochloride [(+)-5**].** Obtained from 5-acetyl-2-furoyl chloride³⁶ (0.10 g, 0.61 mmol) and (–)-**12** free base (0.20 g, 0.58 mmol): 0.12 g (42%); mp 246–249 °C; R_f = 0.51 (eluting mixture B); $[\alpha]_D^{20}$ +110.2 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.30–2.08 (m, 7H, H_{5–8} octahydroquinoxaline), 2.38–2.55 (m, 4H, H_{5–8} octahydroquinoxaline and CH₃), 3.78–4.30 (m, 10H, H_{2–3} octahydroquinoxaline, OCH₃), 4.38–4.50 (m, 1H, H_{4a} octahydroquinoxaline), 4.62–4.80 (m, 1H, H_{8a} octahydroquinoxaline), 7.25 (m, 1H, H₃ furan), 7.50–7.62 (m, 2H, H₄ furan and aromatics), 7.80 (s, 1H, aromatics), 8.65 (br s, 1H, NH, exchangeable with D₂O), 8.90 (br s, 1H, NH, exchangeable with D₂O), 12.05 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₅H₂₉N₅O₅·HCl·3.25H₂O: C, 52.26; H, 6.40; N, 12.19. Found: C, 52.02; H, 6.61; N, 12.12.

6.1.8.5. (+)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-4-(2-thienylcarbonyl)octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine hydrochloride [(+)-6**].** Obtained from thiophene-2-carbonyl chloride (0.089 g, 0.61 mmol) and (–)-**12** free base (0.2 g, 0.58 mmol): 0.06 g (21%); mp 280–282 °C; R_f = 0.42 (eluting mixture B); $[\alpha]_D^{20}$ +46.4 (*c* 1, MeOH); ^1H NMR (DMSO- d_6): δ 1.25–2.10 (m, 7H, H_{5–8} octahydroquinoxaline), 2.25–2.48 (m, 1H, H_{5–8} octahydroquinoxaline), 3.70–4.22 (m, 10H, H_{2–3} octahydroquinoxaline, OCH₃), 4.25–4.42 (m, 1H, H_{8a} octahydroquinoxaline), 4.57–4.78 (m, 1H, H_{4a} octahydroquinoxaline), 7.18 (m, 1H, H₄ thiophene), 7.42–7.65 (m, 2H, H_{3,5} thiophene), 7.78 (s, 1H, aromatics), 7.82 (m, 1H, aromatics), 8.62 (br s, 1H, NH, exchangeable with D₂O), 8.90 (br s, 1H, NH, exchangeable with D₂O), 11.90 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₃H₂₇N₅O₃·HCl·2.5H₂O: C, 51.63; H, 6.22; N, 13.09. Found: C, 51.45; H, 6.16; N, 13.04.

6.1.8.6. (+)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-4-(1*H*-pyrrol-2-ylcarbonyl)octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine hydrochloride [(+)-7**].** Obtained from 1*H*-pyrrole-2-carbonyl chloride³⁷ (0.079 g, 0.61 mmol) and (–)-**12** free base (0.2 g, 0.58 mmol): 0.014 g (5%); mp 284–286 °C; R_f = 0.46 (eluting mixture D); $[\alpha]_D^{20}$ +45.0 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.22–2.10 (m, 7H, H_{5–8} octahydroquinoxaline), 2.30–2.45 (m, 1H, H_{5–8} octahydroquinoxaline), 3.70–4.42 (m, 11H, H_{2–3} and H_{8a} octahydroquinoxaline, OCH₃), 4.50–

4.72 (m, 1H, H_{4a} octahydroquinoxaline), 6.18 (m, 1H, H₄ pyrrole), 6.62 (m, 1H, H₃ pyrrole), 6.90 (m, 1H, H₅ pyrrole), 7.50 (s, 1H, aromatics), 7.75 (s, 1H, aromatics), 8.65 (br s, 1H, NH, exchangeable with D₂O), 8.94 (br s, 1H, NH, exchangeable with D₂O), 11.52 (br s, 1H, NH, exchangeable with D₂O), 11.85 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₃H₂₈N₆O₃·HCl·2.75H₂O: C, 52.87; H, 6.66; N, 16.08. Found: C, 53.18; H, 6.53; N, 15.68.

6.1.8.7. (+)-6,7-Dimethoxy-2-[(4a*S*,8a*R*)-4-(pyridin-2-ylcarbonyl)octahydroquinoxalin-1(2*H*)-yl]quinazolin-4-amine dihydrochloride [(+)-8**].** Obtained from pyridine-2-carbonyl chloride³⁸ (0.11 g, 0.76 mmol) and (–)-**12** free base (0.25 g, 0.73 mmol): 0.074 g (19%); mp 287–289 °C; R_f = 0.29 (eluting mixture B); $[\alpha]_D^{20}$ +73.2 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.30–2.15 (m, 7H, H_{5–8} octahydroquinoxaline), 2.25–2.48 (m, 1H, H_{5–8} octahydroquinoxaline), 3.65–4.15 (m, 10H, H_{2–3} octahydroquinoxaline, OCH₃), 4.35–4.42 (m, 1H, H_{8a} octahydroquinoxaline), 4.70–4.90 (m, 1H, H_{4a} octahydroquinoxaline), 7.50–7.80 (m, 4H, H₃, H₅ pyridine and aromatics), 7.92–8.10 (m, 1H, H₄ pyridine), 8.60–8.76 (m, 2H, H₆ pyridine and NH exchangeable with D₂O), 8.95 (br s, 1H, NH, exchangeable with D₂O), 12.05 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₄H₂₈N₆O₃·2HCl·3H₂O: C, 50.09; H, 6.31; N, 14.60. Found: C, 50.18; H, 6.47; N, 14.31.

6.1.8.8. (+)-2-[(4a*S*,8a*R*)-4-Benzoyloctahydroquinoxalin-1(2*H*)-yl]-6,7-dimethoxyquinazolin-4-amine hydrochloride [(+)-9**].** Obtained from benzoyl chloride (0.094 g, 0.67 mmol) and (–)-**12** free base (0.22 g, 0.64 mmol): 0.08 g (26%); mp 281–283 °C; R_f = 0.45 (eluting mixture A); $[\alpha]_D^{20}$ +36.8 (*c* 0.5, MeOH); ^1H NMR (DMSO- d_6): δ 1.28–2.15 (m, 7H, H_{5–8} octahydroquinoxaline), 2.40–2.50 (m, 1H, H_{5–8} octahydroquinoxaline), 3.62–4.12 (m, 10H, H_{2–3} octahydroquinoxaline, OCH₃), 4.22–4.43 (m, 1H, H_{8a} octahydroquinoxaline), 4.62–4.85 (m, 1H, H_{4a} octahydroquinoxaline), 7.38–7.67 (m, 6H, aromatics), 7.75 (s, 1H, aromatics), 8.62 (br s, 1H, NH, exchangeable with D₂O), 8.90 (br s, 1H, NH, exchangeable with D₂O), 12.00 (br s, 1H, NH, exchangeable with D₂O). Anal. Calcd for C₂₅H₂₉N₅O₃·HCl·1.75H₂O: C, 58.25; H, 6.55; N, 13.59. Found: C, 58.16; H, 6.87; N, 13.48.

6.2. Pharmacology

6.2.1. Functional experiments. Tissues for experiments were taken from male Wistar rats (275–300 g; Charles River, Como, Italy). After killing by cervical dislocation, the required organs were isolated. Vas deferens prostatic portion, spleen and aorta were freed from adhering connective tissue and set up rapidly, under a suitable tension, in 20-mL organ baths. The bath medium, containing physiological salt solution (pH 7.4), was kept at 37 °C and aerated with 5% CO₂/95% O₂. Concentration–response curves were constructed by cumulative addition of agonist. The agonist concentration in the bath was increased approximately 3-fold at each step, with each addition being made only after the response of the previous addition had attained a maximal level and remained steady. Contractions were

recorded by means of a force displacement transducer connected to the MacLab System PowerLab/800.

In all experiments a control agonist concentration–response curve (vehicle) was constructed in the presence of the maximum DMSO concentration (0.5%) contained in the bathing solutions being the solvent used for dissolution of tested antagonists on preparing the initial stock solution. These curves were not different from the previous one indicating no interference of solvent in the agonist effect. The agonist-elicited concentration–response curves obtained in the presence of the tested concentrations of antagonist were related to the vehicle control curve, of which the maximal response was taken as 100%. Parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. The experimental conditions used for the investigation at α_1 -adrenoceptor subtypes are procedures taken from the quoted literature.

All pharmacological graphics were drawn by a Prism 3.0 computer program (GraphPad Software, Inc., San Diego, CA, USA). Chemicals, (–)-noradrenaline bitartrate, (–)-phenylephrine hydrochloride, cocaine hydrochloride, normetanephrine hydrochloride and (±)-propranolol hydrochloride were purchased from Sigma–Aldrich Srl (Milano, Italy).

6.2.1.1. Prostatic rat vas deferens. Affinity at α_{1A} adrenoceptor was evaluated on prostatic rat vas deferens according to a reported procedure.²¹ Prostatic portions of 2 cm length were mounted under 0.35 g tension at 37 °C in Tyrode solution of the following composition (mM): NaCl, 130; KCl, 2; CaCl₂, 1.8; MgCl₂, 0.89; NaH₂PO₄, 0.42; NaHCO₃, 25; glucose, 5.6. To prevent the neuronal uptake of noradrenaline, used as agonist, cocaine hydrochloride (10 μ M) was added to the Tyrode solution 20 min before the agonist cumulative concentration–response curve was recorded. Vas deferens was equilibrated for 45 min, with washing every 15 min. After the equilibration period, tissues were primed twice by addition of 10 μ M noradrenaline in order to obtain a constant response. After another washing and equilibration period of 45 min, a cumulative isotonic noradrenaline concentration–response curve was constructed to determine the relationship between agonist concentrations and contractile response. When measuring the effect of the antagonist, it was allowed to equilibrate with the tissue for 30 min before constructing a new concentration–response curve to the agonist. The noradrenaline solution contained 0.05% Na₂S₂O₅ to prevent oxidation.

6.2.1.2. Aorta. Affinity at rat aorta α_{1D} adrenoceptor was evaluated using a procedure adapted from that already reported.²² Two strips (15 mm $\times$ 3 mm) were cut helically from rat thoracic aorta beginning from the end most proximal to the heart. The endothelium was removed by rubbing with filter paper: the absence of 100 μ M acetylcholine-induced relaxation to preparations contracted with 1 μ M noradrenaline was taken as an indicator that the vessel was denuded successfully. The strips were then tied with

surgical thread and suspended in an organ bath containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; CaCl₂, 1.9; MgSO₄, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11.7. Cocaine hydrochloride (10 μ M), normetanephrine hydrochloride (1 μ M) and propranolol hydrochloride (1 μ M) were added to prevent the neuronal and extraneuronal uptake of the agonist noradrenaline and to block the β -adrenoceptors, respectively. In the absence of these inhibitors the noradrenaline concentration–response curve was significantly displaced to the right (data not shown).

After an equilibration period of at least two hours under an optimal tension of 1 g, cumulative noradrenaline concentration–response curves were recorded isometrically at 1 h intervals, the first being discarded and the second one taken as control. After inspection of vehicle activity, the antagonist was allowed to equilibrate with the tissue for 30 min before generation of the third cumulative concentration–response curve to the agonist. Noradrenaline solutions contained 0.05% K₂EDTA in 0.9% NaCl to prevent oxidation.

6.2.1.3. Spleen. The spleen was removed and bisected longitudinally in two strips, which were suspended in tissue baths containing Krebs solution of the following composition (mM): NaCl, 120; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.5; KH₂PO₄, 1.2; NaHCO₃, 20; glucose, 11; EDTA, 0.01. Propranolol hydrochloride (4 μ M) was added to block β -adrenoceptors. Following a reported procedure the spleen strips were placed under 1 g resting tension and equilibrated for 2 h.²⁴ A first cumulative concentration–response curve to the agonist phenylephrine was quickly taken isometrically, followed by 30 min washing. Subsequently, a second cumulative curve was constructed followed by 30 min washing. Each tissue was then incubated either with vehicle or different antagonist concentrations and a new agonist concentration–response curve constructed.

To test the reproducibility in the tissue, a new concentration–response curve to phenylephrine, after the second, was repeated at different successive times, that is, 30, 45 and 60 min, corresponding to different possible periods of incubation with the antagonist. The third concentration–response curve, recorded after 30 min, did not modify significantly either the pD₂ value or the maximum response of the agonist, whereas a significant decrease of the maximum response from the control curve was observed after 45 and 60 min (34% and 41%, respectively).

On the basis of these results, a time of 30 min was selected as the period for antagonist incubation. As a consequence, measuring the antagonism of (+)-cyclazosin and related new compounds, the various compound concentrations were equilibrated for 30 min with tissue before constructing the new phenylephrine concentration–response curve (third curve).

6.2.2. Binding assays. Competition binding assays to cloned human α_{1a} , α_{1b} and α_{1d} -adrenoceptor subtypes

were performed in membrane preparations from CHO (Chinese hamster ovary) cell lines transfected by electroporation with DNA expressing the gene encoding each α_1 -adrenoceptor. Cloning and stable expression of the human α_1 -adrenoceptor gene was performed as previously described.²⁷ Briefly, CHO cell membranes (30 μ g proteins) were incubated in 50 mM Tris–HCl buffer, pH 7.4, with 0.1–0.4 nM [³H]prazosin, in a final volume of 1.02 mL for 30 min at 25 °C, in the absence or presence of competing drugs (1 pM–10 μ M). Non-specific binding was determined in the presence of 10 μ M phen-tolamine. The incubation was stopped by addition of ice-cold Tris–HCl buffer and rapid filtration through 0.2% poly(ethylenimine)-pretreated Whatman GF/B or Schleicher & Schuell GF52 filters.

6.2.3. Data analysis. In functional studies, responses were expressed as percentage of the maximal contraction observed in the agonist concentration–response curve taken as control. Each response was plotted graphically as a mean from at least four separate experiments. Curves were fitted to all the data by a non-linear regression using the Prism 3.0 program to calculate pEC₅₀ values. In all cases, 50% of the maximum for each concentration–response curve was used to evaluate the EC₅₀. This value, calculated in presence and in absence of antagonist in a single tissue, was used to determine the concentration ratio.

Schild plots were constructed to estimate the pA₂ values and the slope of the regression line using experimental series obtained from at least three different concentrations.²⁵ The Schild diagrams were constructed by plotting the log (concentration ratio – 1) against the log [antagonist] and deriving it from a linear regression using the Prism 3.0 program. When the Schild plot slope was not significantly different from unity ($p > 0.05$), the regression was recalculated with a constrained slope of 1 and the result given as a pA₂ value. In a number of cases, Schild analysis could not be performed due to the non-parallel slopes of concentration–response curves and variable depression. As a consequence, pK_B values were calculated at only one concentration, according to van Rossum.²⁶

Thus, a pA₂ value was determined for (+)-1, (+)-3, (+)-4 and (+)-9 at the α_{1A} -adrenoceptor, for (+)-3 at the α_{1B} -adrenoceptor and for (+)-1 - (+)-6 at the α_{1D} -adrenoceptor. In all other cases the antagonist potency was expressed by pK_B values calculated with the equation $pK_B = \log (\text{concentration ratio} - 1)/[\text{antagonist}]$ at the lowest antagonist concentration giving a significant rightward shift (≥ 0.5) of the agonist concentration–response curve.

Data were compared by Student's *t*-test and presented as means $\pm$ SEM of 4–6 experiments. A *p* value < 0.05 was taken to indicate a statistically significant difference.

Data from binding assays were analysed using a non-linear curve-fitting program Allfit.³⁹ Scatchard plots were linear in all preparations and the pseudo-Hill coefficients non-significantly different from the unity ($p > 0.05$). The inhibition of the radioligand specific binding by tested compounds al-

lowed the estimation of IC₅₀ values that were converted to affinity constants (*K_i*) by the Cheng–Prusoff equation²⁸: $K_i = IC_{50}/(1 + L/K_d)$, where *L* and *K_d* are the concentration and the equilibrium dissociation constant of the radioligand. Data are expressed as means of p*K_i* values of 2–3 separate experiments performed in triplicate.

Acknowledgments

The present work was supported by a grant from the University of Camerino and Cofinanziamento MIUR 2005 (Italy).

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