

RAD: 7-104255- -20	FECHA: 2013-09-26 16:17:14
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 5
ACT: 519 PRESENTACION	

Abogado(a)/Señor(a)
LUZ CLEMENCIA SUAREZ DE PAEZ

Asunto:	Radicación:	7-104255- -20
	Trámite:	11
	Evento:	378
	Actuación:	519
	Oficio:	18504
	Folios:	5

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/JP2006/307047				
Fecha de Presentación Internacional	03/04/2006				

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Rad. N°07-104255-00000-0000	04/Octubre/2007
Reivindicaciones:	Rad. N°07-104255-00000-0000	04/Octubre/2007
	Rad. N°07-104255-00010-0000	30/Abril/2012
Dibujos (Figuras) :	Rad. N°07-104255-00000-0000	04/Octubre/2007
Oposiciones:	Rad. N°07-104255-0000 -0000	Lafrancol 30/Julio/2008
	Rad. N°07-104255-0000 -0000	Gynopharm S.A. 30/Julio/2008
Respuesta del solicitante:	Rad. N°07-104255-00008 -0000	09/diciembre/2008
Prioridad:	JP 2005 107674	04/abril/2005

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

Título de la solicitud: “MEDIDA DE PREVENCIÓN O REMEDIO PARA NEUROSIS POR DEPRESIÓN O ANSIEDAD”

El problema planteado en esta solicitud consiste en la necesidad de proporcionar un agente profiláctico o terapéutico para trastornos por depresión o ansiedad con baja toxicidad.

Para resolver este problema técnico la solicitud en estudio proporciona una composición que comprende combinar el compuesto (S)-N-[2-(1, 6,7,8-tetrahidro-2H-inden[5,4-b]furan -8-il)etil] propionamida y uno o más fármacos seleccionados de (según radicado N° 07-104255-00010-0000).

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 31/343, C07D 307/93, A61P 25/24.

4. De la Solicitud de Patente

Titulo

El título no corresponde con el objeto de la solicitud porque no menciona la composición objeto de la invención.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La Reivindicación 1 no es concisa porque da origen a un gran número de composiciones, lo cual hace difícil definir cuál es el objeto que se busca proteger y además de todas estas composiciones no existe sustento en la descripción.

Descripción (Art 28 D 486)

La Descripción no es suficiente porque la información que contiene no permite que la persona del oficio normalmente versada en la materia pueda comprender el problema técnico y la solución aportada por la invención. Además de todas estas composiciones no existe sustento en la descripción sólo se evidencia el ejemplo de la tabla 1 (pág 5) que se refiere a una tableta que comprende el compuesto A.

Se requiere al Solicitante hacer las aclaraciones necesarias.

5. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a mínimo 2 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

1° Composición que comprende (S)-N-[2-(1, 6, 7,8-tetrahidro-2H-inden [5,4-b]furan -8-il)etil] propionamida y Diazepam.

2° Composición (S)-N-[2-(1, 6, 7, 8-tetrahidro -2H-inden [5,4-b] furan -8-il)etil] propionamida y Paroxetina.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

6. Análisis de las Oposiciones

Las sociedades opositoras Lafrancol S.A. y Gynopharm S.A. argumentan que hay reivindicaciones que se refieren al uso y a un método de tratamiento y no sólo a la composición farmacológica o a un método de fabricación mediante el cual se obtenga un producto tangible, que según el artículo 14 de la Decisión 486 de la Comunidad Andina corresponde a materia que no es patentable.

La sociedad opositora Lafrancol S.A. afirma que esta solicitud pretende obtener patente para un nuevo uso de la sustancia (S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida (Ramelteon), empleado actualmente en la prevención o tratamiento de trastornos de depresión o ansiedad, resaltando que la sustancia (S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida (Ramelteon), reivindicada en la solicitud colombiana en trámite, corresponde a un fármaco en primera instancia indicado y aprobado en el tratamiento de trastornos del sueño (insomnio), tal y como lo demuestra la patente americana US 6.024.239 publicada el 07 de marzo de 2000 y titulada *"Tricyclic compounds, their production and use"*.

Por su parte, la sociedad opositora Gynopharm S.A. afirma que antes de la fecha de prioridad de esta solicitud se encuentra el documento ES2175350T, publicado el 16 de noviembre de 2002, que afecta la patentabilidad de la presente solicitud. El documento revela un compuesto de fórmula (I), en la que R<1> es un hidrocarburo, amino o grupo heterociclo opcionalmente sustituido; R<2> es H o un grupo hidrocarburo opcionalmente sustituido; R 3 es H o un hidrocarburo o grupo heterocíclico opcionalmente sustituido, X es CHR<4>, NR<4>, O o S en la cual R<4> es H o un hidrocarburo opcionalmente sustituido; Y es C, CH o N; el anillo A es un anillo de 5 a 7 eslabones opcionalmente sustituidos; el anillo B es un anillo de benceno opcionalmente sustituido; y m es 1 a 4, o una sal del mismo. Agrega, además, que el documento divulga en la página 75, líneas 38 a página 76, línea 50, que el compuesto (I) es altamente selectivo, especialmente frente al receptor ML-1. Adicionalmente, el compuesto tiene baja toxicidad, pocos efectos secundarios y actúa como agonista de la melatonina en mamíferos, por lo cual, se puede usar para prevenir y curar trastornos del control biorítmico y otros trastornos que pueden ser controlados por la melatonina. Según este documento, el compuesto (I) de la composición se puede usar con otros ingredientes activos (por ejemplo fármacos del tipo benzodiazepina), por lo que concluye que el objeto se deriva, evidentemente, a partir de dicha anterioridad.

Frente a tales argumentos, la solicitante modificó el capítulo reivindicatorio de tal manera que no se observan reivindicaciones relacionadas con usos y métodos de tratamiento. Asimismo, la solicitante señaló que en la solicitud se encuentra el compuesto A (Ramelteon), que presenta una acción antidepresiva y antiansiedad mayor que la de la melatonina y que mediante la combinación de un compuesto A con otro compuesto específico de acción antidepresiva o antiansiedad conocido en el estado del arte, tales como los revelados en la presente solicitud, se obtiene un efecto antidepresivo y antiansiedad mejorado.

De acuerdo con lo expuesto y frente al nuevo capítulo reivindicatorio aportado, esta Oficina encuentra que el documento US6034239 (que es equivalente al documento ES2175350T) enseña el compuesto (S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida y su posible combinación con fármacos antidepresivos y anti-ansiedad, por esta razón, este documento desvirtúa el nivel inventivo del objeto de esta solicitud (composiciones propuestas, por lo tanto se declaran fundadas las oposiciones).

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la Solicitud de Prioridad: 04 de abril de 2005, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Nº	Documento	Título	Fecha de Publicación*
D1	US6034239	Tricyclic compounds, their production and use	07/03/2000

D2	Artículo ²	Combined effects of diazepam and melatonin in two tests for anxiolytic activity in the mouse	1992
D3	WO2005/002562	The combination of a serotonin reuptake inhibitors and agomelatine	13/01/2005

D1¹ el compuesto (S)-N-[2-(1, 6, 7, 8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida (ramelteon) , con una excelente afinidad de unión por los receptores de melatonina en composiciones farmacéuticas, junto con otros fármacos antidepresivos y antiansiedad como la benzodiazepinas y como el diazepam.

D2² divulga el efecto combinado, con actividad ansiolítica, del Diazepam y melatonina.

D3³ la combinación de agomelatina (modulador de MT1) y un compuesto inhibidor de la recaptación de serotonina, para el tratamiento de la depresión y otros desórdenes afectivos.

8 . Examen de Patentabilidad (Art 14 D486)

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en Composición farmaceutica que comprende combinar (según radicado N°07-104255-00010-0000). Se analiza el 1° grupo inventivo.

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

Características esenciales	D1	D2
Composición farmacéutica que comprende combinar:	Composición farmacéutica (columna 146, ejemplo de formulación 3).	Combinación que comprende
(S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan- 8-il)etil] propionamida uno o más fármacos	(S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida (columna 30, líneas 64-65; columna 134, líneas 29-52).	No menciona (S)-N-[2-(1,6,7,8-tetrahidro-2H-inden[5,4-b]furan- 8-il)etil] propionamida sino otro modulador de los receptores de melatonina Melatonina
Diazepam.	Fármacos tipo benzodiazepinas como el diazepam (columna 104, líneas 37- 40)	Diazepam

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D1 divulga el compuesto (S)-N-[2-(1, 6, 7, 8-tetrahidro-2H-inden[5,4-b]furan-8-il)etil]propionamida (ramelteon) , con una excelente afinidad de unión por los receptores de melatonina (col.1 campo técnico, col. 30, ln. 64-65; col. 134, ln. 29-52) en composiciones farmacéuticas, junto con otros fármacos antidepresivos y antiansiedad como la benzodiazepinas y como el diazepam (col. 104, ln. 37-40).

La diferencia entre la invención, definida en la reivindicación 1 y la composición del documento D1 consiste en que no se presenta específicamente una composición con el componente adicional al (S)-N-[2-(1, 6, 7,8-tetrahidro-2H- inden [5,4-b] furan-8-il) etil] propionamida.

1[http://worldwide.espacenet.com/searchResults?](http://worldwide.espacenet.com/searchResults?compact=false&PN=US6034239&ST=advanced&locale=en_EP&DB=EPODOC)

[compact=false&PN=US6034239&ST=advanced&locale=en_EP&DB=EPODOC](http://worldwide.espacenet.com/searchResults?compact=false&PN=US6034239&ST=advanced&locale=en_EP&DB=EPODOC)

2 Anexo al concepto.

3[http://worldwide.espacenet.com/searchResults?](http://worldwide.espacenet.com/searchResults?compact=false&PN=WO2005002562&ST=advanced&locale=en_EP&DB=EPODOC)

[compact=false&PN=WO2005002562&ST=advanced&locale=en_EP&DB=EPODOC](http://worldwide.espacenet.com/searchResults?compact=false&PN=WO2005002562&ST=advanced&locale=en_EP&DB=EPODOC)

El efecto causado por la inclusión de un componente con actividad terapéutica adicional es el aumento en la actividad ansiolítica y antidepresiva respecto del ramelteon cuando está solo.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: ¿cómo proporcionar composiciones con mayor actividad ansiolítica y antidepresiva que incluyan ramelteon?

Sin embargo, el documento D1 ya sugería la utilización del ramelteon en combinación con benzodiacepinas, como el diazepam (col. 104, ln. 37-40).

De otro lado, D2 enseña el efecto combinado con actividad ansiolítica, del Diazepam y melatonina (afinidad de unión por los receptores de melatonina). Además, ya se conocía que el (S)-N-[2-(1, 6, 7, 8-tetrahidro-2H-inden [5,4- b] furan-8-il) etil] propionamida (ramelteon) es un compuesto con mayor afinidad de unión por los receptores de melatonina, que la melatonina⁴ (pág. 4229).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el diazepam de D2 en la composición divulgada en D1 para llegar así al objeto de la reivindicación 1 de la solicitud, sin que esto muestre ningún efecto inesperado ya que el arte previo lo enseñaba claramente. Por lo cual se considera obvia.

Puesto que las reivindicación dependiente 2 no menciona ninguna característica inventiva adicional tampoco se puede considera inventiva.

Respecto al segundo grupo inventivo, de la reivindicación 1, el documento D3 revela la combinación de la agomelatina (agonista del receptor metaloninérgico MT1) y un compuesto inhibidor de la recaptación de serotonina, para el tratamiento de la depresión y otros desórdenes afectivos en donde se enseña que el compuesto inhibidor de la recaptación de serotonina puede ser la Paroxetina, Fluoxetina, sertalina, milnacipran, Citalopram, escitalopram, Duloxetina, Venlafaxina, imipramina, clomipramina, trazodona (pág. 8 ln.3, reivindicaciones 1, 7, 15, 24, pág.15); de manera que la persona versada en la materia tendría motivación suficiente para incluir el compuesto (S)-N-[2-(1, 6, 7, 8-tetrahidro-2H-inden [5,4- b] furan-8-il) etil] propionamida (ramelteon) de D1, en la combinación de D3 con la expectativa de llegar al objeto de la composición del segundo grupo inventivo o sea la composición de ramelteon y Paroxetina, sin que esto muestre ningún efecto inesperado ya que el arte previo lo enseñaba claramente. Por lo cual la composición reivindicad se considera obvia.

Puesto que las reivindicación dependiente 2 no menciona ninguna característica inventiva adicional tampoco se puede considera inventiva.

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Nº	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US6034239	1-2	Nivel Inventivo
D2	Artículo ²	1-2	Nivel Inventivo
D3	WO2005/002562	1-2	Nivel Inventivo

⁴ Anexo al concepto

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 2013-10-01

Elaboró: Lyna Del Carmen Mordecay Dunoyer
Revisó: Gloria Jacqueline Alfonso Roza

Neurochemical properties of ramelteon (TAK-375), a selective MT₁/MT₂ receptor agonist

Koki Kato^a, Keisuke Hirai^a, Keiji Nishiyama^a, Osamu Uchikawa^b,
Kohji Fukatsu^b, Shigenori Ohkawa^b, Yuji Kawamata^c,
Shuji Hinuma^c, Masaomi Miyamoto^{a,d,*}

^aPharmacology Research Laboratories I, Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd.,
Osaka 532-8686, Japan

^bMedicinal Chemistry Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd.,
Osaka 532-8686, Japan

^cDiscovery Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd.,
Osaka 300-4293, Japan

^dStrategic Product Planning Department, Takeda Pharmaceutical Company Ltd., 4-1-1 Doshomachi, Chuo-ku,
Osaka 540-8645, Japan

Received 28 April 2004; received in revised form 6 September 2004; accepted 16 September 2004

Abstract

Ramelteon (TAK-375) is a novel melatonin receptor agonist currently under investigation for the treatment of insomnia. This study describes the neurochemical and receptor binding characteristics of ramelteon *in vitro*. Ramelteon showed very high affinity for human MT₁ (Mel_{1a}) and MT₂ (Mel_{1b}) receptors (expressed in Chinese hamster ovary [CHO] cells), and chick forebrain melatonin receptors (consisting of Mel_{1a} and Mel_{1c} receptors) with *K_i* values of 14.0, 112, and 23.1 pM, respectively, making the affinities of ramelteon for these receptors 3–16 times higher than those of melatonin. The affinity of ramelteon for hamster brain MT₃ binding sites was extremely weak (*K_i*: 2.65 μM) compared to melatonin's affinity for the MT₃ binding site (*K_i*: 24.1 nM). In addition, ramelteon showed no measurable affinity for a large number of ligand binding sites (including benzodiazepine receptors, dopamine receptors, opiate receptors, ion channels, and transporters) and no effect on the activity of various enzymes. Ramelteon inhibited forskolin-stimulated cAMP production in the CHO cells that express the human MT₁ or MT₂ receptors. Taken together, these results indicate that ramelteon is a potent and highly selective agonist of MT₁/MT₂ melatonin receptors.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Ramelteon (TAK-375); Melatonin; MT₁ receptor; MT₂ receptor; Sleep disorder; Insomnia

1. Introduction

Melatonin, a hormone secreted by the pineal gland, is thought to be involved in regulating sleep-wake cycles in humans and other mammals. Melatonin production varies according to a circadian rhythm

controlled by neuronal output from the suprachiasmatic nucleus (SCN) of the hypothalamus. The production of melatonin in humans is concurrent with nocturnal sleep, and the increase in melatonin levels in the evening correlates with the onset of self-reported evening sleepiness (Akerstedt et al., 1979; Zhdanova et al., 1996) and an increase in sleep propensity (Tzischinsky et al., 1993).

Melatonin exerts its action through membrane-associated receptors (for review, see Vanecek, 1998).

* Corresponding author. Tel.: +81 6 62042337; fax: +81 6 62042344.

E-mail address: miyamoto_masaomi@takeda.co.jp (M. Miyamoto).

Initial radioligand studies revealed putative melatonin receptors in both neuronal and non-neuronal tissues from vertebrate species. 2-[¹²⁵I]Melatonin binding sites were originally defined as high-affinity binding sites (picomolar affinity, in chick and mammalian brains and retina) and low-affinity binding sites (nanomolar affinity, in mammalian brains and peripheral organs) (Dubocovich, 1995; Reppert et al., 1996). Two high-affinity human melatonin receptors have been cloned: MT₁ (also designated as Mel_{1a}, Reppert et al., 1994; Slaugenhaupt et al., 1995) and MT₂ (also designated as Mel_{1b}, Reppert et al., 1995a) receptors nomenclatured by IUPHAR (Dubocovich et al., 2000). The MT₁ and MT₂ receptors in the SCN are thought to be involved in the effect of melatonin on circadian rhythms (Dubocovich et al., 2003; von Gall et al., 2002).

The low-affinity binding site (MT₃, Dubocovich, 1995; Paul et al., 1999) has a profile that is completely different from those of the MT₁ and MT₂ receptors (Nosjean et al., 2000, 2001). It has recently been characterized as a melatonin-sensitive form of quinone reductase 2 (QR2), an enzyme related to the detoxifying enzyme NAD(P)H:quinone oxidoreductase 1 (QR1, EC 1.6.99.2), and it shows a wide tissue distribution, including kidney, liver, brain, heart and skeletal muscle in mammals.

Because of melatonin's involvement in natural sleep, the use of exogenous melatonin has been examined as a treatment for sleep disorders. Exogenous melatonin has been shown to have a direct sleep-promoting action (Zhdanova et al., 1995, 2001), however, it has not been shown to have any consistent effect on total sleep time or sleep efficiency (Hughes et al., 1998; Zhdanova and Wurtman, 1997). Also, melatonin may have therapeutic effects on circadian rhythm sleep disorders including jet lag and shift-work sleep disorder (Arendt et al., 1995; Sharkey et al., 2001). The secretion of endogenous melatonin declines with aging, and this may be related to the sleep-wake rhythm disturbance in elderly people (Mirmiran et al., 1992; Skene et al., 1990). Abnormal decreases in nocturnal melatonin have also been observed in patients with Alzheimer's disease, another population prone to sleep disturbances (Mirmiran et al., 1992; Skene et al., 1990).

Ramelteon, (S)-N-[2-(1,6,7,8-tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]propionamide (TAK-375), is a novel melatonin receptor agonist under investigation for the treatment of insomnia. In animal models, ramelteon has a sleep-promoting action and does not appear to cause learning, memory, or motor function impairment, or to have rewarding properties (Miyamoto et al., 2003, 2004). In a clinical study, ramelteon decreased latency to persistent sleep and increased total sleep time and sleep efficiency in subjects with primary chronic insomnia as measured by polysomnography (Erman et al., 2003). The present study was done to

characterize the neurochemical and receptor binding properties of ramelteon as a way to distinguish it from melatonin and other sleep agents that act at different receptors.

2. Materials and Methods

2.1. High-affinity melatonin receptor binding assays

Receptor binding assays were based on the methodology of Rivkees et al. (1989) with minor modification. For the chick melatonin receptor binding assay, fore-brains from 7-day-old male chicks (Komatsu, Japan) were homogenized with 20–30 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) and centrifuged at 44 000×g for 10 min at 4 °C. The resulting pellet was washed twice and stored at –30 °C until use in the binding assays. Test compound and 80 pM 2-[¹²⁵I]melatonin were mixed with the thawed homogenate in a total volume of 500 µL and incubated at 25 °C for 90 min. The reaction was terminated by addition of 3 mL of ice-cold buffer followed by vacuum filtration on a Whatman GF/B. The filter was washed twice and radioactivity was counted by a γ-counter. Nonspecific binding was defined as the binding in the presence of 10 µM melatonin.

For the human MT₁ receptor binding assay, cDNA encoding the human MT₁ gene was introduced into Chinese hamster ovary (CHO) cells. A cell line stably expressing MT₁ receptors (CHO-hMelR7) was selected and cultured in Eagle's Minimum Essential Medium-α (MEM-α) supplemented with 10% dialyzed fetal bovine serum (dFBS) under a 5% CO₂/95% air atmosphere. Cells were harvested at confluence in Ca²⁺–Mg²⁺ free Hanks' balanced salt solution containing 5 mM EDTA and collected by centrifugation. Cells were homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C), washed twice, pelleted, and stored at –30 °C until the binding assays were conducted. Test compound and 40 pM 2-[¹²⁵I]melatonin were mixed with the thawed homogenate in a total volume of 1 mL and incubated at 25 °C for 150 min. The reaction was terminated by addition of 3 mL of ice-cold buffer followed by vacuum filtration on a Whatman GF/B. The filter was washed twice and radioactivity was counted by a γ-counter. Nonspecific binding was defined as the binding in the presence of 10 µM melatonin.

For the human MT₂ receptor binding assay, CHO cells were transfected with a pCMV-human MT₂ receptor expression vector using LipofectAMINE reagent (Invitrogen). A cell line stably expressing MT₂ receptors was selected and cultured in MEM-α supplemented with 10% FBS and 300 µg/mL geneticin (Invitrogen) under a 5% CO₂/95% air atmosphere.

Cells expressing MT₂ receptors were harvested at confluence in Dulbecco's Phosphate-Buffered Saline and collected by centrifugation. Cells were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.6 at 25 °C), washed twice, pelleted, and stored at -80 °C until the binding assays were conducted. Test compound was mixed with the thawed homogenate and 280 pM 2-[¹²⁵I]melatonin in a total volume of 1 mL and incubated at 25 °C for 150 min. The reaction was terminated by addition of 3 mL of ice-cold buffer followed by vacuum filtration on a Whatman GF/B. The filter was washed twice and radioactivity was counted by a γ -counter. Nonspecific binding was defined as the binding in the presence of 100 μ M melatonin.

The 50% inhibitory concentration (IC₅₀) was calculated using the nonlinear logistic regression analysis. The dissociation constant of the compound for the receptor (K_i) was calculated using following equation:

$$K_i = IC_{50} / (1 + L/K_d)$$

where *L* and *K_d* represent the concentration and the dissociation constant of 2-[¹²⁵I]melatonin in the binding assay, respectively.

2.2. MT₃ binding assay

The procedure for this binding assay was based on the methodology of Niles et al. (1987) with minor modification. Brains were dissected from 7- to 8-week-old male Syrian hamsters (SLC, Japan) and stored in 0.32 M sucrose at -30 °C until use. The brain tissue was homogenized with 20–30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 4 °C) and centrifuged at 48 000 $\times$ g for 10 min at 4 °C. The pellet was washed twice and suspended in buffer for use in the binding assay. Test compound (25 μ L) was mixed with hamster brain homogenate and 100 pM 2-[¹²⁵I]melatonin in a total volume of 250 μ L and incubated at 4 °C for 60 min. The reaction was terminated by the addition of 3 mL of ice-cold buffer followed by vacuum filtration on a Whatman GF/B. The filter was washed twice and radioactivity was counted using a γ -counter. Nonspecific binding was defined as the binding in the presence of 100 μ M melatonin. K_i values were calculated as described above.

2.3. cAMP production in CHO cells expressing the human MT₁ or MT₂ receptor

A single clone of CHO-hMeIR7 exhibiting specific 2-[¹²⁵I]melatonin binding and the inhibition of forskolin-stimulated cAMP production by melatonin (CHO-hMeIR7-58) was selected by the limiting dilution method and cultured in MEM- α /10% dFBS, harvested at confluence by trypsin/EDTA treatment and seeded

at 5 $\times$ 10⁴ cells/well in 12-well plates (Iwaki glass). After culturing for 3 days, cells were washed twice with 1 mL of modified Hanks' balanced salt solution (mHBSS: HBSS containing 10 mM HEPES-Na [pH 7.3] instead of 4.16 mM NaHCO₃) at 37 °C, and 0.5 mL of mHBSS containing 100 μ M 3-isobutyl-1-methylxanthine and a test compound were added followed by preincubation at 37 °C for 6 min. After this, 10 μ L of 50 μ M forskolin in mHBSS was added (final concentration 1 μ M), and the cells were incubated an additional 15 min. The reaction was terminated by the addition of 50 μ L of 55% perchloric acid. A 200- μ L aliquot of the reaction mixture was neutralized with 100 μ L of 1.6 M NaOH and assayed for cAMP content using a cAMP [¹²⁵I] assay system (Amersham Pharmacia Biotech Ltd. Buckinghamshire, UK).

A subclone (CHO-hMT2 #13), which shows inhibition of forskolin-stimulated cAMP production by melatonin, was selected using the limiting dilution method in the presence of 500 μ g/mL geneticin. CHO-hMT2 #13 was cultured in MEM- α containing 10% FBS and 500 μ g/mL geneticin, harvested at confluence by trypsin-EDTA treatment and seeded at 4 $\times$ 10⁴ cells/well in 96-well plates (Asahi Techno Glass, Tokyo, Japan). After culturing for 1 day, cells were washed twice with 200 μ L modified Hanks' balanced salt solution (HBSS-HEPES, HBSS containing 20 mM HEPES buffer [pH 7.3]); 215 μ L of HBSS-HEPES containing 10 μ M 3-isobutyl-1-methylxanthine (HBSS-IBMX) and a test compound were added followed by preincubation at 37 °C for 6 min. After this, 10 μ L of forskolin solution in HBSS-IBMX was added (final concentration 1 μ M), and the cells were incubated for 15 min. The reaction was terminated by the addition of 25 μ L of lysis buffer and a 100- μ L aliquot of the reaction mixture was assayed for cAMP content using a cAMP Enzyme immunoassay Biotark system (Amersham Pharmacia Biotech Ltd. Buckinghamshire, UK).

The IC₅₀ was calculated using the nonlinear logistic regression analysis.

2.4. Assays for other receptors and enzymes

Effects of ramelteon and melatonin on various receptors, ion channels and transporters were evaluated using standardized assay protocols (MDS Pharma Services, Taipei, Taiwan). Receptors, transporters and ion channels tested are shown in Table 1. Initially, ramelteon and melatonin were tested for inhibition of binding at 10 μ M. Further investigation was performed using multiple concentrations of ramelteon and melatonin to calculate the IC₅₀ value when inhibition exceeded 50% at 10 μ M.

Table 1

List of receptors for which ramelteon did not inhibit binding more than 50% at 10 μ M

Receptor	Receptor	Receptor
Adenosine A ₁	Epidermal growth factor (EGF)	Neurokinin NK ₁
Adenosine A _{2A}	Estrogen	Neurokinin NK ₂
Adenosine A ₃	GABA transporter	Neurokinin NK ₃
Adenosine transporter	GABA _A	Neuropeptide Y ₁
Adrenergic α_{1A}	GABA _A (benzodiazepine, central)	Neuropeptide Y ₂
Adrenergic α_{1B}	GABA _A (benzodiazepine, peripheral)	Neurotensin
Adrenergic α_1	GABA _A (chloride channel)	Nicotinic (central)
Adrenergic α_{2A}	GABA _B	Opiate δ
Adrenergic α_{2B}	Galanin	Opiate κ
Adrenergic α_{2C}	Glucagon-like peptide-1 (GLP-1)	Opiate μ
Adrenergic α_2	Glucocorticoid	Opiate (non-selective)
Adrenergic β_1	Glutamate (AMPA)	Phorbol ester
Adrenergic β_2	Glutamate (kainate)	Platelet activating factor (PAF)
Adrenergic β_3	Glutamate (NMDA)	Platelet derived growth factor (PDGF)
Adrenergic β (non-selective)	Glutamate (NMDA, glycine)	Potassium channel (K _A)
Adrenergic NE transporter	Glutamate (NMDA, PCP)	Potassium channel (K _{ATP})
Angiotensin AT ₁	Glutamate (NMDA, polyamine)	Potassium channel (K _v)
Angiotensin AT ₂	Glutamate (non-selective)	Potassium channel (SK _{Ca})
Atrial natriuretic factor (ANF)	Glycine (strychnine)	Progesterone
Bombesin	Histamine H ₁ (central)	Purinergic P _{2x}
Bradykinin B ₁	Histamine H ₁ (peripheral)	Rolipram
Bradykinin B ₂	Histamine H ₂	Serotonin 5-HT ₁ (non-selective)
Calcitonin	Histamine H ₃	Serotonin 5-HT _{1A}
Calcitonin gene related peptide (CGRP)	Imidazoline I ₂ (central)	Serotonin 5-HT _{1B}
Calcium channel (L type, diltiazem)	Imidazoline I ₂ (peripheral)	Serotonin 5-HT ₂
Calcium channel (L type, nitrendipine)	Inositol triphosphate (IP ₃)	Serotonin 5-HT ₃
Calcium channel (L type (-) D888)	Insulin	Serotonin 5-HT ₄
Calcium channel (N type)	Interferon γ	Serotonin 5-HT _{5A}
Cannabinoid CB ₁	Interleukin (IL-1 α)	Serotonin 5-HT ₆
Cannabinoid CB ₂	Interleukin (IL-2)	Serotonin 5-HT ₇
Cholecystokinin (CCKA)	Interleukin (IL-6)	Serotonin transporter
Cholecystokinin (CCKB)	Interleukin (IL-8)	Sigma σ_1
Choline transporter	CXCR1 (IL-8 _A)	Sigma σ_2
Dopamine D ₁	CXCR2 (IL-8 _B)	Sigma (non-selective)
Dopamine D _{2L}	Leukotriene (LTB ₄)	Sodium channel (site 2)
Dopamine D _{2S}	Leukotriene (LTD ₄)	Somatostatin
Dopamine D ₃	Monoamine transporter	Testosterone
Dopamine D _{4.2}	Muscarinic M ₁	Thromboxane A ₂
Dopamine D _{4.4}	Muscarinic M ₂	Thyrotropin releasing hormone (TRH)
Dopamine D _{4.7}	Muscarinic M ₃	Transforming growth factor- β 1 (TGF- β)
Dopamine D ₅	Muscarinic M ₄	Tumor necrosis factor- α (TNF- α)
Dopamine transporter	Muscarinic M ₅	Vasointestinal peptide (VIP ₁)
Endothelin ET _A	Muscarinic (non-selective)	Vasopressin V ₁
Endothelin ET _B	Muscarinic (oxotremorine-M)	

Effects of ramelteon and melatonin on various enzymes were examined by conventional methods using various concentrations of ramelteon and melatonin (MDS Pharma Services, Taipei, Taiwan). Tested enzymes and concentrations of compounds are shown in Table 2.

2.5. Materials

Ramelteon, (*S*)-*N*-[2-(1,6,7,8-tetrahydro-2*H*-indeno-[5,4-*b*]furan-8-yl)ethyl]propionamide (TAK-375, Fig. 1), was synthesized at Takeda Chemical Industries, Ltd. (Osaka, Japan) (Uchikawa et al., 2002). Melatonin and *N*-acetylserotonin (*N*-acetyl-5-HT) were purchased from

Sigma–Aldrich (St. Louis, MO), and 2-iodomelatonin was obtained from Tocris Cookson Ltd. (Bristol, UK). Other substances were purchased from Wako Pure Chemical (Osaka, Japan). All test compounds were dissolved in dimethylsulfoxide (DMSO) to make a 10 mM solution and diluted in assay solutions. The final concentration of DMSO was less than 1% in assay solutions. Treatment with vehicle, which contained DMSO, had no significant effects on binding assays, cAMP production and enzyme assays. 2-[¹²⁵I]Melatonin was obtained from Perkin Elmer Inc. (Wellesley, MA). The cAMP [¹²⁵I] assay system and cAMP Enzyme immunoassay Biotark system were obtained from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK).

Table 2
List of enzymes for which the inhibition by ramelteon and melatonin were examined

Enzyme	Compound (μM)	Enzyme	Compound (μM)
Acetyl CoA synthetase	100	15-Lipoxygenase	30
Acetylcholinesterase	10	Monoamine oxidase A	10
Acyl CoA-cholesterol acyltransferase (hepatic)	100	Monoamine oxidase B	10
Acyl CoA-cholesterol acyltransferase (intestine)	100	Myeloperoxidase	10
Aldose reductase	100	Neutral endopeptidase	10
Angiotensin converting enzyme	10	Nitric oxide synthase, constitutive (cNOS)	10
ATPase- H^+/K^+ (gastric)	30	Nitric oxide synthase, inducible (iNOS)	10
Ca^{2+} /Calmodulin-dependent protein kinase II	100	Phosphodiesterase type 1 (PDE1)	100
Calcineurin (PP2B tyrosine phosphatase)	10	Phosphodiesterase type 2 (PDE2)	100
Calpain	10	Phosphodiesterase type 3 (PDE3)	100
Carbonic anhydrase	10	Phosphodiesterase type 4 (PDE4)	100
Cathepsin G	30	Phosphodiesterase type 5 (PDE5)	100
CD45 tyrosine phosphatase	10	Phospholipase A_2	300
Choline acetyltransferase	100	Phospholipase C	10
Collagenase IV	100	p59fyn tyrosine kinase	10
Cyclooxygenase-1	300	pS6lck tyrosine kinase	10
Cyclooxygenase-2	300	Protein kinase A, non-selective	100
EGF receptor tyrosine kinase	10	Protein kinase C, non-selective	300
Elastase	30	Protein kinase C- α	100
Free radical scavenger, SOD mimetic	10	Protein kinase C- β (I and II)	100
HER2 tyrosine kinase	10	Protein kinase C- γ	100
HMG CoA reductase	30	Protein tyrosine phosphatase 1B	10
β -Lactamase	100	Protein tyrosine phosphatase 1C	100
Leukotriene A_4 hydrolase	100	T cell tyrosine phosphatase	10
Leukotriene C_4 synthetase	1000	Thromboxane A_2 synthetase	100
Lipid peroxidase	300	Tyrosine hydroxylase	10
5-Lipoxygenase	30	Xanthine oxidase	10

Effects of ramelteon and melatonin on various enzyme activities were tested at concentrations shown.

Abbreviation: EGF, epidermal growth factor; HMG-CoA, 3 hydroxy-3-methylglutaryl coenzyme A; SOD, superoxide dismutase.

3. Results

3.1. Affinity for the human MT_1/MT_2 receptors and chick melatonin receptors

The specific binding of 2- ^{125}I melatonin in the chick forebrain, which contain the chick Mel_{1a} and Mel_{1c} receptors (Reppert et al., 1995b), was saturable. The Scatchard plot of the saturation isotherm using ligand concentrations ranging from 10 to 160 pM revealed high-affinity binding sites with a K_d value of 24.7 ± 2.3 pM and a B_{max} of 13.2 ± 0.9 fmol/mg protein (three experiments). This was also the case for human MT_1 receptors in CHO-hMelR7, with a K_d value of 15.0 ± 3.0 pM and a B_{max} of 555 ± 114 fmol/mg protein (three

experiments), determined with ligand concentrations ranging from 5 to 80 pM. Human MT_2 receptors expressed in CHO cells showed lower affinities than those of human MT_1 receptors, and its K_d value and B_{max} were 328 ± 12 pM and 133 ± 2 fmol/mg protein, respectively (three experiments) determined with ligand concentrations ranging from 99 to 6350 pM.

The affinities of the tested compounds for these receptors are shown in Table 3. The affinity of ramelteon for the chick melatonin receptors was found to be very high, with a K_i value of 23.1 pM, which is comparable to that of 2-iodomelatonin and 16 times higher than that of melatonin. *N*-Acetyl-5-HT, a precursor of melatonin with high affinity for the MT_3 binding site, showed very low affinity for high-affinity melatonin receptors including the MT_1 , MT_2 , and chick melatonin receptors. Prazosin, an α_1 antagonist with high affinity for the MT_3 binding site (Niles et al., 1987; Pickering and Niles, 1990), showed no affinity for the high-affinity melatonin receptors.

All compounds showed 2- to 4-fold higher affinity for the human MT_1 receptor than for the chick melatonin receptors with almost the same order of potency. The affinity of ramelteon for human MT_1 was comparable to that of 2-iodomelatonin and 6 times higher than that of melatonin (Fig. 2 and Table 3). Ramelteon and 2-iodomelatonin also showed high affinity for the

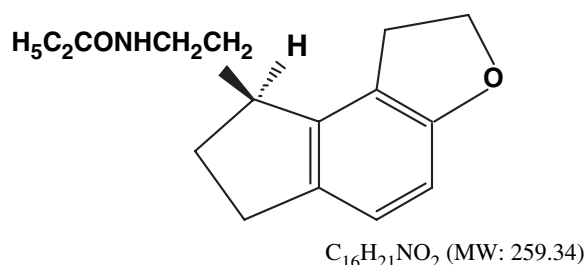


Fig. 1. Chemical structure of ramelteon.

Table 3

Affinities of ramelteon and other compounds on the human MT₁/MT₂ receptors and chick melatonin receptors

Compound	K_i (pM)		
	Chick forebrain (Mel _{1a} + Mel _{1c})	CHO-hMelR7 (MT ₁)	CHO (MT ₂)
Ramelteon	23.1 ± 0.4	14.0 ± 0.5	112 ± 5
Melatonin	368 ± 9	80.7 ± 2.1	383 ± 5
2-Iodomelatonin	24.8 ± 1.7	13.1 ± 0.3	188 ± 4
<i>N</i> -acetyl-5-HT	583 000 ± 22 000	81 300 ± 6 900	3 640 000 ± 30 000
Prazosin	> 2 360 000	> 2 730 000	> 5 370 000

Chick forebrain melatonin receptors consist of the chick Mel_{1a} and Mel_{1c} receptors. Each value represents the mean of three experiments with the S.E.M.

human MT₂ receptor with K_i values of 112 and 188 pM, respectively (Fig. 2 and Table 3). Compared to their affinities for the MT₁ receptor, ramelteon and 2-iodomelatonin showed lower affinity for the MT₂ receptor. *N*-Acetyl-5-HT and prazosin showed little or no affinity for human MT₁ and MT₂ receptors.

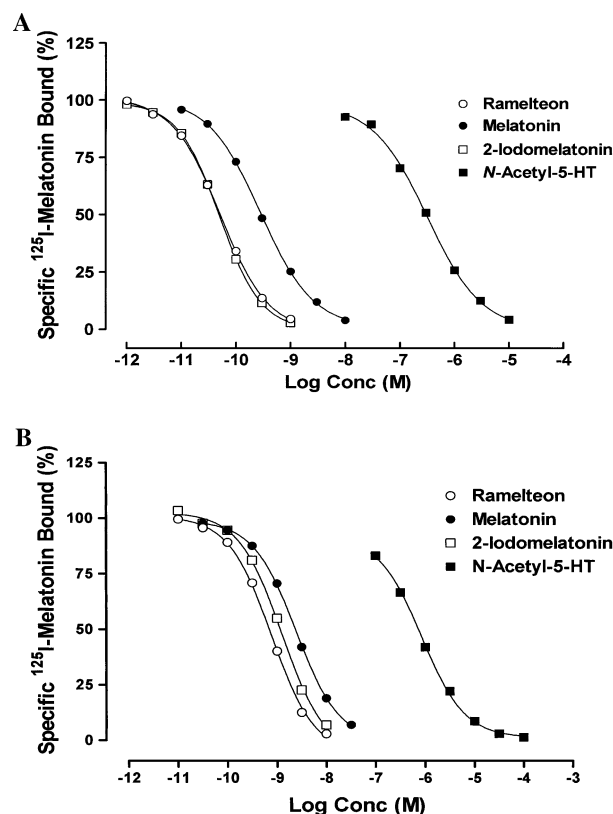


Fig. 2. Competition by various ligands for 2-[¹²⁵I]melatonin binding to CHO cells expressing the human MT₁ (A) and MT₂ (B) receptors. (A) Cell homogenate of CHO cells expressing the human MT₁ receptor were incubated with 40 pM 2-[¹²⁵I]melatonin and various concentrations of ramelteon, melatonin, 2-iodomelatonin and *N*-acetyl-5-HT. Nonspecific binding was determined in the presence of 10 μM melatonin. (B) Cell homogenate of CHO cells expressing the human MT₂ receptor were incubated with 280 pM 2-[¹²⁵I]melatonin and various concentrations of ramelteon, melatonin, 2-iodomelatonin and *N*-acetyl-5-HT. Nonspecific binding was determined in the presence of 100 μM melatonin.

3.2. Affinity for the MT₃ binding site

The specific binding of 2-[¹²⁵I]melatonin to the hamster MT₃ binding site was saturable. The Scatchard plot of the saturation isotherm using ligand concentrations ranging from 100 to 2500 pM revealed a single binding site component with a K_d value of 900 ± 89 pM and a B_{max} of 80.3 ± 6.6 fmol/mg protein (three experiments). The affinity of the radioligand for these receptors was about 1/40 of that for the chick melatonin receptors (Fig. 3).

The affinities of compounds for the hamster MT₃ binding site are summarized in Table 4. Ramelteon showed very low affinity for the MT₃ binding sites with a K_i value of 2.65 μM, which was 1/115 000 of its affinity for the chick melatonin receptors. In contrast, both melatonin and 2-iodomelatonin demonstrated high affinities for the MT₃ binding site. The affinity of ramelteon for the MT₃ site was 1/110 that of melatonin and 1/2750 that of 2-iodomelatonin. *N*-acetyl-5-HT and prazosin also showed high affinities for the MT₃ binding site, as reported previously (Dubocovich, 1995; Niles et al., 1987). The affinities of these two compounds were higher than that of melatonin for the MT₃ binding site and higher than the affinities of these compounds for the chick melatonin receptors. Our results indicate that ramelteon is highly selective for the high-affinity melatonin receptors in contrast to other compounds.

3.3. Inhibition of forskolin-induced cAMP production in CHO cells expressing the human MT₁ or MT₂ receptor

The stimulation of CHO-hMelR7-58 cells with 1 μM forskolin elevated cAMP content to 6- to 12-fold above basal level. Ramelteon, melatonin, and 2-iodomelatonin inhibited forskolin-stimulated cAMP production in a concentration-dependent manner (Fig. 4A). Table 5 shows IC₅₀ values of these compounds. These results indicate that ramelteon is a potent agonist to human MT₁ receptor, as potent as 2-iodomelatonin and about 3.7 times more potent than melatonin.

The stimulation of CHO-hMT2 #13 cells with 1 μM forskolin potentially elevated cAMP content above basal

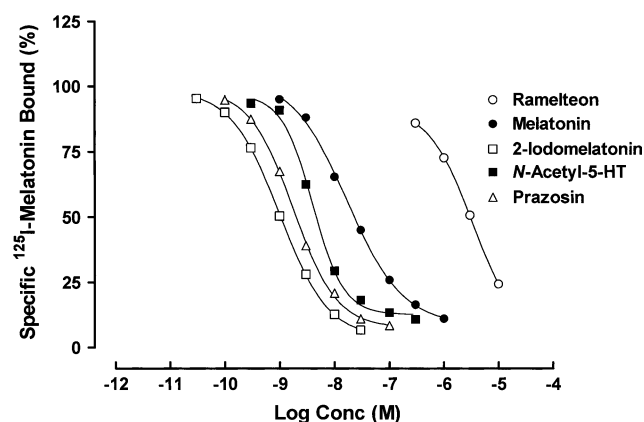


Fig. 3. Competition by various ligands for 2-[¹²⁵I]melatonin binding to the *MT₃* binding site in hamster brain. Hamster brain homogenate was incubated with 100 pM 2-[¹²⁵I]melatonin and various concentrations of ramelteon, melatonin, 2-iodomelatonin, *N*-acetyl-5-HT and prazosin at 4 °C for 60 min. Nonspecific binding was determined in the presence of 100 μM melatonin.

level. Ramelteon, melatonin, and 2-iodomelatonin inhibited forskolin-stimulated cAMP production in a concentration-dependent manner (Fig. 4B). Table 6 shows *IC₅₀* values of these compounds. Ramelteon and 2-iodomelatonin potently inhibited forskolin-stimulated cAMP production in the cells with *IC₅₀* of 53.4 and 60.7 pM, respectively. Melatonin showed moderate inhibition with *IC₅₀* of 904 pM. These results indicate that ramelteon is an agonist to human *MT₂* receptor, as potent as 2-iodomelatonin and about 17 times more potent than melatonin.

3.4. Effects on various receptors and enzymes

Ramelteon did not show significant (>50%) inhibition of the binding of any receptors, transporters, and ion channels listed in Table 1 at 10 μM, including monoamine receptors, opioid receptors, central benzodiazepine receptors, and dopamine transporters. Melatonin caused no significant inhibition of the binding of almost all receptors, except for 5-HT_{1A} receptor, for which the *K_i* value was 5.6 μM. Moreover, ramelteon and melatonin had no effect on the activity of any enzymes listed in Table 2 at 10–1000 μM.

4. Discussion

In the present study, ramelteon demonstrated high affinity to high-affinity melatonin receptors, including human *MT₁* and *MT₂* receptors. The *K_i* of ramelteon was 3–16 times higher than that of endogenous ligand melatonin, and this is consistent with pharmacological effects of ramelteon and melatonin in vivo. Previously, we showed that ramelteon exerts a sleep-promoting action in freely moving cats (Miyamoto et al., 2004) and accelerates re-entrainment of circadian rhythm of running-wheel activity following an abrupt phase-advance in rats. These effects were more potent than those of melatonin (data not shown).

Ramelteon inhibited forskolin-stimulated cAMP production in CHO cells expressing the human *MT₁* and *MT₂* melatonin receptors, which indicates that ramelteon is an *MT₁*/*MT₂* receptor agonist. While melatonin inhibited forskolin-stimulated cAMP production with an *IC₅₀* value comparable to the *K_i* value for the *MT₁* receptor binding, ramelteon and 2-iodomelatonin showed *IC₅₀* values slightly higher than the *K_i* value. This suggests that the endogenous ligand melatonin has slightly higher efficacy in the stimulation of the *MT₁* receptors than the synthetic ligands. It has been shown that activation of the *MT₁* or *MT₂* receptor by melatonin inhibits forskolin-induced cAMP formation via pertussis toxin-sensitive G protein (Morgan et al., 1989; Reppert et al., 1994, 1995a), which is consistent with the present results. Melatonin also inhibits forskolin-stimulated cAMP-dependent activation of protein kinase A activity (Morgan et al., 1994) and phosphorylation of cAMP response element binding protein (McNulty et al., 1994). Thus, a cAMP-dependent signal transduction cascade may be involved in ramelteon-induced pharmacological effects.

Activation of *MT₁* and *MT₂* receptors in the SCN are closely related to melatonin-induced hypnotic and chronobiotic effects (for review, see Dubocovich et al., 2003; von Gall et al., 2002). In mice with a target deletion of the *MT₁* receptor, the acute inhibitory effect of melatonin on neuronal firing rate in the SCN was abolished, while the melatonin-mediated phase-shifting effect appeared normal (Liu et al., 1997). The

Table 4
Affinity of ramelteon and other compounds on the *MT₃* binding sites in the hamster brain

Compound	<i>K_i</i> (nM)		Ratio <i>MT₃</i> /(<i>Mel_{1a}</i> + <i>Mel_{1c}</i>)
	Hamster brain (<i>MT₃</i>)	Chick forebrain (<i>Mel_{1a}</i> + <i>Mel_{1c}</i>)	
Ramelteon	2650 ± 180	0.0231 ± 0.0004	115 000
Melatonin	24.1 ± 0.5	0.368 ± 0.009	65.5
2-Iodomelatonin	0.964 ± 0.015	0.0248 ± 0.0018	38.9
<i>N</i> -acetyl-5-HT	15.7 ± 2.8	583 ± 22	0.0269
Prazosin	6.16 ± 0.46	> 2360	–

Chick forebrain melatonin receptors consist of the chick *Mel_{1a}* and *Mel_{1c}* receptors. Each value represents the mean of three experiments with the S.E.M.

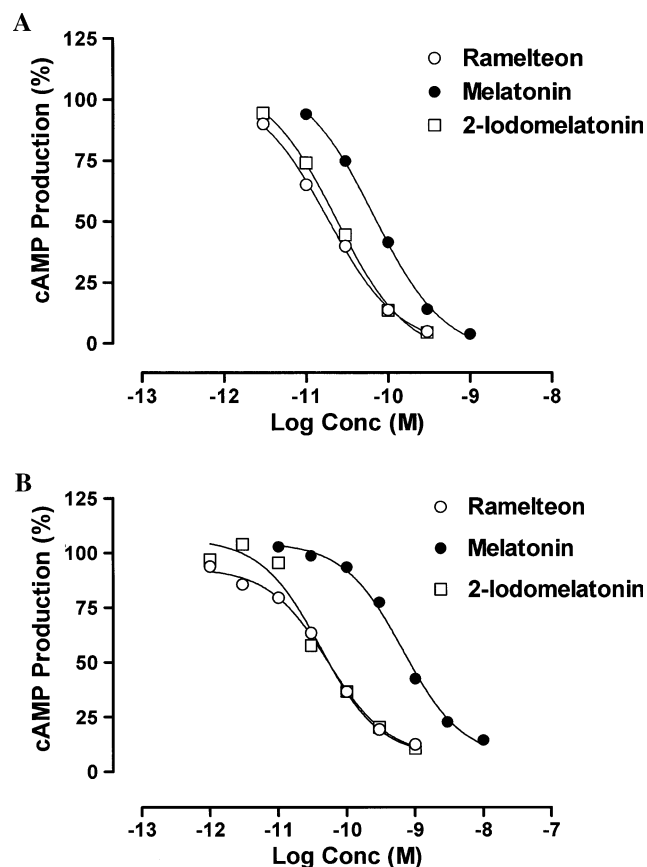


Fig. 4. Effects of ramelteon on forskolin-induced cAMP production in CHO cells expressing the human MT_1 (A) or MT_2 (B) receptors. The 100% value is the mean cAMP production with 1 μ M forskolin. The data are mean values of 3–4 experiments (A) or two experiments (B) conducted in triplicate.

suppression of the neuronal activity by melatonin might be important defining the SCN sensitivity to entraining stimuli, and in diurnal species may contribute to the regulation of sleep (von Gall et al., 2002). On the other hand, MT_2 receptor antagonists have been shown to block melatonin-mediated phase advances of circadian rhythms (Dubocovich et al., 1998).

In mammals, melatonin regulates reproductive and neuroendocrine functions (Reiter, 1980; Malpaux et al., 2001). Although most studies on the reproductive function of melatonin have been focused in the hypothalamus and pituitary, recently it has been shown that MT_1 and MT_2 melatonin receptors are expressed in rat ovaries dependent on the stage of the estrous cycle and may affect steroidogenesis through cAMP mediated signaling (Soares et al., 2003). Furthermore, the MT_1 receptors have been shown to express in normal and malignant human breast tissue, and melatonin inhibits the growth of breast cancer cells (Dillon et al., 2002; Yuan et al., 2002). It has also been reported that melatonin regulates vascular tone. The MT_1 and MT_2 melatonin receptors are present in rat caudal arteries

Table 5

Effects of ramelteon and other compounds on forskolin-stimulated cAMP production in CHO cells expressing the human MT_1 receptors

Compound	IC ₅₀ (pM)	cAMP production
Ramelteon	21.2 ± 0.5	
Melatonin	77.8 ± 14.6	
2-Iodomelatonin	26.8 ± 7.5	

Each value represents the mean of 3–4 experiments with the S.E.M.

(Masana et al., 2002). Activation of the MT_1 receptors facilitate adrenergic vasoconstriction (Krause et al., 1995; Ting et al., 1997), while activation of the MT_2 receptors mediate vasorelaxation (Masana et al., 2002). Ramelteon shows high affinity for the MT_1 and MT_2 receptors; thus, these potential peripheral effects of ramelteon should be investigated by further studies.

Ramelteon showed a low affinity for MT_3 , the binding site identified as human QR2 (Nosjean et al., 2000). Good correlation between the amount of the MT_3 binding sites and QR2 reductase activity has been observed in various organs (including brain, liver, kidney and heart) in the mouse and dog, although apparent discrepancies were seen in monkey tissues (Nosjean et al., 2001). In a previous study (Miyamoto et al., 2003), we found that melatonin and *N*-acetyl-5-HT, a precursor of melatonin biosynthesis and a potent ligand of the MT_3 binding site (Popova and Dubocovich, 1995), potentiated the diazepam-induced deficit of rota-rod performance in mice, whereas ramelteon did not show any such effect, suggesting that this action of melatonin might be related to action at the MT_3 binding site. Correlation between melatonin- and *N*-acetyl-5-HT-induced aggravation of diazepam-induced motor deficits and their effect on QR2 activity is not clear. The potential for this action of melatonin to cause unfavorable side effects when used clinically is not clear; however, these facts suggest that a selective MT_1/MT_2 agonist would be preferable to a non-selective melatonin agonist for clinical treatment of sleep disturbance.

The neurochemical profile of ramelteon, which includes a negligible affinity for central benzodiazepine receptors, opioid receptors, dopamine transporters, and other receptors and enzymes tested even at high concentrations, is consistent with results of our previous

Table 6

Effects of ramelteon on forskolin-stimulated camp production in CHO cells expressing the human MT_2 receptors

Compound	IC ₅₀ (pM)
Ramelteon	53.4 (40.7–70.3)
Melatonin	904 (714–1150)
2-Iodomelatonin	60.7 (44.0–83.9)

Each IC₅₀ value is calculated from two experiments done in triplicate. Numbers in parentheses indicate 95% confidence intervals of IC₅₀ values.

pharmacological studies. Ramelteon had no effects on motor function tested by Rota-rod performance in mice or on learning and memory as demonstrated by the water-maze task and delayed matching to position tasks in rats (Miyamoto et al., 2003). We have studied the abuse potential of ramelteon by the place-preference test in rats and found that the compound, like melatonin, has a very low potential for abuse, unlike morphine and benzodiazepines (Miyamoto et al., 2003).

In conclusion, ramelteon is a potent and highly selective MT₁/MT₂ melatonin receptor agonist. Its neurochemical and binding characteristics suggest that it has the potential to be a useful therapeutic agent for the treatment of insomnia.

References

- Akerstedt, T., Fröberg, J.E., Friberg, Y., Wetterberg, L., 1979. Melatonin excretion, body temperature, and subjective arousal during 64 hours of sleep deprivation. *Psychoneuroendocrinology* 4, 219–225.
- Arendt, J., Deacon, S., English, J., Marks, V., Arendt, J.H., 1995. Melatonin and adjustment to phase shift. *Journal of Sleep Research* 4, 74–79.
- Dillon, D.C., Easley, S.E., Asch, B.B., Cheney, R.T., Brydon, L., Jockers, R., Winston, J.S., Hurd, T., Asch, H.L., 2002. Differential expression of high-affinity melatonin receptor (MT₁) in normal and malignant human breast tissue. *American Journal of Clinical Pathology* 118, 451–458.
- Dubocovich, M.L., 1995. Melatonin receptors: are there multiple subtypes? *Trends in Pharmacological Sciences* 16, 50–56.
- Dubocovich, M.L., Yun, K., Al-Ghoul, W.M., Benloucif, S., Masana, M.I., 1998. Selective MT₂ melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. *FASEB Journal* 12, 1211–1220.
- Dubocovich, M.L., Cardinali, D.P., Delagrange, P., Krause, D.N., Stroberg, D., Sugden, D., Yocca, F.D., 2000. In: *Melatonin Receptors. The IUPHAR Compendium of Receptor Characterization Classification*. IUPHAR Media, London, pp. 270–277.
- Dubocovich, M.L., Rivera-Bermudez, M.A., Gerdin, M.J., Masana, M.I., 2003. Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Frontiers in Bioscience* 8, d1093–d1108.
- Erman, M., Seiden, D., Zammit, G., 2003. Phase II study of the selective MT₁ receptor agonist TAK-375 in subjects with primary chronic insomnia. *Sleep* 26, A298.
- Hughes, R.J., Sack, R.L., Lewy, A.J., 1998. The role of melatonin and circadian phase in age-related sleep-maintenance insomnia: assessment in a clinical trial of melatonin replacement. *Sleep* 21, 52–68.
- Krause, D.N., Barioss, V.E., Duckles, S.P., 1995. Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery. *European Journal of Pharmacology* 276, 207–213.
- Liu, C., Weaver, D.R., Jin, X., Shearman, L.P., Pieschl, R.L., Gribkoff, V.K., Reppert, S.M., 1997. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* 19, 91–102.
- Malpau, B., Migaud, M., Tricoire, H., Chemineau, P., 2001. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. *Journal of Biological Rhythms* 16, 336–347.
- Masana, M.I., Doolen, S., Ersahin, C., Al-Ghoul, W.M., Duckles, S.P., Dubocovich, M.L., Krause, D.N., 2002. MT₂ melatonin receptors are present and functional in rat caudal artery. *Journal of Pharmacology and Experimental Therapeutics* 302, 1295–1302.
- McNulty, S., Ross, A.W., Barrett, P., Hastings, M.H., Morgan, P.J., 1994. Melatonin regulates the phosphorylation of CREB in ovine pars tuberalis. *Journal of Neuroendocrinology* 6, 523–532.
- Mirmiran, M., Swaab, D.F., Kok, J.H., Hofman, M.A., Witting, W., van Gool, W.A., 1992. Circadian rhythms and the suprachiasmatic nucleus in perinatal development, aging and Alzheimer's disease. *Progress in Brain Research* 93, 151–163.
- Miyamoto, M., Nishikawa, H., Ohta, H., Uchikawa, O., Ohkawa, S., 2003. Behavioral pharmacology of TAK-375 in small animals. *Annals of Neurology* 54 (Suppl. 7), S46.
- Miyamoto, M., Nishikawa, H., Doken, Y., Hirai, K., Uchikawa, O., Ohkawa, S., 2004. The sleep-promoting action of ramelteon (TAK-375) in freely moving cats. *Sleep*, in press.
- Morgan, P.J., Lawson, W., Davidson, G., Howell, H.E., 1989. Guanine nucleotides regulate the affinity of melatonin receptors on the ovine pars tuberalis. *Neuroendocrinology* 50, 359–362.
- Morgan, P.J., Barrett, P., Howell, H.E., Helliwell, R., 1994. Melatonin receptors: localization, molecular pharmacology and physiological significance. *Neurochemistry International* 24, 101–146.
- Niles, L.P., Pickering, D.S., Sayer, B.G., 1987. HPLC-purified 2-[¹²⁵I]iodomelatonin labels multiple binding sites in hamster brain. *Biochemical and Biophysical Research Communications* 147, 949–956.
- Nosjean, O., Ferro, M., Cogé, F., Beauverger, P., Henlin, J.M., Lefoulon, F., Fauchère, J.L., Delagrange, P., Canet, E., Boutin, J.A., 2000. Identification of the melatonin binding site MT₃ as the quinone reductase 2. *Journal of Biological Chemistry* 275, 31311–31317.
- Nosjean, O., Nicolas, J.-P., Klupsch, F., Delagrange, P., Canet, E., Boutin, J.A., 2001. Comparative pharmacological studies of melatonin receptors: MT₁, MT₂ and MT₃/QR2. Tissue distribution of MT₃/QR2. *Biochemical Pharmacology* 61, 1369–1379.
- Paul, P., Lahaye, C., Delagrange, P., Nicolas, J.P., Canet, E., Boutin, J.A., 1999. Characterization of 2-[¹²⁵I]iodomelatonin binding sites in Syrian hamster peripheral organs. *Journal of Pharmacology and Experimental Therapeutics* 290, 334–340.
- Pickering, D.S., Niles, L.P., 1990. Pharmacological characterization of melatonin binding sites in Syrian hamster hypothalamus. *European Journal of Pharmacology* 175, 71–77.
- Popova, J.S., Dubocovich, M.L., 1995. Melatonin receptor-mediated stimulation of phosphoinositide breakdown in chick brain slices. *Journal of Neurochemistry* 64, 130–138.
- Reiter, R.J., 1980. The pineal and its hormones in the control of reproduction in mammals. *Endocrinology Review* 1, 109–131.
- Reppert, S.M., Weaver, D.R., Ebisawa, T., 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* 13, 1177–1185.
- Reppert, S.M., Godson, C., Mahle, C.D., Weaver, D.R., Slaugenhaupt, S.A., Gusella, J.F., 1995a. Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel_{1b} melatonin receptor. *Proceedings of the National Academy of Sciences of the United States of America* 92, 8734–8738.
- Reppert, S.M., Weaver, D.R., Cassone, V.M., Godson, C., Kolakowski, L.F., 1995b. Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron* 15, 1003–1015.
- Reppert, S.M., Weaver, D.R., Godson, C., 1996. Melatonin receptors step into the light: cloning and classification of subtypes. *Trends in Pharmacological Sciences* 17, 100–102.
- Rivkees, S.A., Cassone, V.M., Weaver, D.R., Reppert, S.M., 1989. Melatonin receptor in chick brain: characterization and localization. *Endocrinology* 125, 363–368.
- Sharkey, K.M., Fogg, L.F., Eastman, C.I., 2001. Effects of melatonin administration on daytime sleep after simulated night shift work. *Journal of Sleep Research* 10, 181–192.

- Skene, D.J., Vivien-Roels, B., Sparks, D.L., Hunsaker, J.C., Pevet, P., Ravid, D., Swaab, D.F., 1990. Daily variation in the concentration of melatonin and 5-methoxytryptophol in the human pineal gland: effect of age and Alzheimer's disease. *Brain Research* 528, 170–174.
- Slaugenhaupt, S.A., Roca, A.L., Liebert, C.B., Altherr, M.R., Gusella, J.F., Reppert, S.M., 1995. Mapping of the gene for the Mel_{1a}-melatonin receptor to human chromosome 4 (MTNR1A) and mouse chromosome 8 (Mtnr1a). *Genomics* 27, 355–357.
- Soares Jr., J.M., Masana, M.I., Ersahin, C., Dubocovich, M.L., 2003. Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. *Journal of Pharmacology and Experimental Therapeutics* 306, 694–702.
- Ting, K., Dunn, W., Davies, D., Sugden, D., Delagrange, P., Guardiola-Lemaitre, B., Scalbert, E., Wilson, V.G., 1997. Studies on the vasoconstrictor action of melatonin and putative melatonin receptor ligands in the tail artery of juvenile Wistar rats. *British Journal of Pharmacology* 122, 1299–1306.
- Tzischinsky, O., Shlitzner, A., Lavie, P., 1993. The association between the nocturnal sleep gate and nocturnal onset of urinary 6-sulfatoxymelatonin. *Journal of Biological Rhythms* 8, 199–209.
- Uchikawa, O., Fukatsu, K., Tokunoh, R., Kawada, M., Matsumoto, K., Imai, Y., Hinuma, S., Kato, K., Nishikawa, H., Hirai, K., Miyamoto, M., Ohkawa, S., 2002. Synthesis of a novel series of tricyclic indan derivatives as melatonin receptor agonists. *Journal of Medicinal Chemistry* 45, 4222–4239.
- Vanecek, J., 1998. Cellular mechanisms of melatonin action. *Physiological Review* 78, 687–721.
- von Gall, C., Stehle, J.H., Weaver, D.R., 2002. Mammalian melatonin receptors: molecular biology and signal transduction. *Cell Tissue Research* 309, 151–162.
- Yuan, L., Collins, A.R., Dai, J., Dubocovich, M.L., Hill, S.M., 2002. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. *Molecular and Cellular Endocrinology* 192, 147–156.
- Zhdanova, I.V., Wurtman, R.J., 1997. Efficacy of melatonin as a sleep-promoting agent. *Journal of Biological Rhythms* 12, 644–650.
- Zhdanova, I.V., Wurtman, R.J., Lynch, H.J., Ives, J.R., Dollins, A.B., Morabito, C., Matheson, J.K., Schomer, D.L., 1995. Sleep-inducing effects of low doses of melatonin ingested in the evening. *Clinical Pharmacology Therapeutics* 57, 552–558.
- Zhdanova, I.V., Wurtman, R.J., Morabito, C., Piotrovskaya, V.R., Lynch, H.J., 1996. Effects of low oral doses of melatonin, given 2–4 hours before habitual bedtime, on sleep in normal young humans. *Sleep* 19, 423–431.
- Zhdanova, I.V., Wurtman, R.J., Regan, M.M., Taylor, J.A., Shi, J.P., Leclair, O.U., 2001. Melatonin treatment for age-related insomnia. *Journal of Clinical Endocrinology and Metabolism* 86, 4727–4730.

Synthesis of a Novel Series of Tricyclic Indan Derivatives as Melatonin Receptor Agonists

Osamu Uchikawa,* Kohji Fukatsu, Ryosuke Tokunoh, Mitsuru Kawada, Kiyoharu Matsumoto, Yumi Imai, Shuji Hinuma, Koki Kato, Hisao Nishikawa, Katsuke Hirai, Masaomi Miyamoto, and Shigenori Ohkawa

Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan

Received March 12, 2002

To develop a new therapeutic agent for sleep disorders, we synthesized a novel series of tricyclic indan derivatives and evaluated them for their binding affinity to melatonin receptors. In our previous paper, we proposed a conformation of the methoxy group favorable for the binding of the MT₁ receptor. To fix the methoxy group in an active conformation, we decided to synthesize conformationally restricted tricyclic indan analogues with the oxygen atom in the 6-position incorporated into a furan, 1,3-dioxane, oxazole, pyran, morpholine, or 1,4-dioxane ring system. Among these compounds, indeno[5,4-*b*]furan analogues were found to be the most potent and selective MT₁ receptor ligands and to have superior metabolic stability. The optimization of substituents led to (S)-(-)-22b, which showed very strong affinity for human MT₁ (*K*_i = 0.014 nM), but no significant affinity for hamster MT₂ (*K*_i = 2600 nM) or other neurotransmitter receptors. The pharmacological effects of (S)-(-)-22b were studied in experimental animals, and it was found that a dose of 0.1 mg/kg, po promoted a sleep in freely moving cats, as demonstrated by a decrease in wakefulness and increases in slow wave sleep and rapid eye movement sleep, which lasted for 6 h after administration. Melatonin (1 mg/kg, po) also had a sleep-promoting effect, though it lasted only 2 h. A new chiral method for the synthesis of (S)-(-)-22b starting from 60, which was prepared from 59 employing asymmetric hydrogenation with the (S)-2,2'-bis(diphenylphosphino)-1,1'-binaphthyl-Ru complex, was developed. (S)-(-)-22b (TAK-375) is currently under clinical trial for the treatment of insomnia and circadian rhythm disorders.

Introduction

The pineal hormone melatonin (1) is of considerable interest for its transduction of photoperiodic information,¹ control of mammalian circadian rhythms,² and modulation of retinal physiology.³ The synthesis of melatonin and its secretion at night from the pineal gland are controlled by a circadian clock within the hypothalamic suprachiasmatic nucleus (SCN)⁴ and are synchronized by environmental light.⁵ Melatonin production in humans is concurrent with nocturnal sleep, and the increase in melatonin levels in the evening correlates with the onset of self-reported evening sleepiness or with the increase in the evening sleep propensity.⁶

In humans, it has been suggested that melatonin might have a variety of clinical applications such as the treatment of delayed sleep phase syndrome,⁷ jet lag,⁸ shift work disturbances,⁹ seasonal affective disorders,¹⁰ blindness, and aging¹¹ and as a hypnotic agent.¹² Over the past few years, numerous reports have described the effects of melatonin on the immune system suggesting that melatonin has immunomodulatory properties.¹³ Additionally, melatonin was shown to inhibit the proliferation of various types of cancer cells and the human breast cancer cell line MCF-7.¹⁴ However, the use of melatonin as a drug is limited by its short biological half-life (15–20 min),¹⁵ its poor oral bioavailability, and its ubiquitous action. Melatonin receptors are classified

as MT₁, MT₂, and MT₃ based on pharmacological profiles. The MT₁ receptor¹⁶ is localized in the hypothalamic SCN and is thought to mediate the circadian and reproductive actions of melatonin, while the MT₂ receptor¹⁷ is distributed in the SCN and neural retina and is thought to mediate the effects of melatonin on circadian rhythms and is implicated in the regulation of visual function. The binding site of the MT₃ receptor has been recently characterized as the hamster homologue of the human enzyme quinone reductase 2.¹⁸ The physiological function of the MT₃ receptor in the brain is not yet completely clear; however, in consideration of therapy for circadian rhythm disorders in the elderly, effects on MT₃ receptors might induce unknown side effects. Because the MT₁ receptor was initially suggested to mediate circadian functions in mammals due to the high level of expression within brain and the MT₂ receptor was expressed in retina and to a lesser extent the brain, we have attempted to develop a MT₁ agonist, which has a longer half-life and is more potent than melatonin but has no affinity for the MT₃ receptor.

Previously, we synthesized a novel series of benzocycloalkene derivatives, with an *endo* double bond, an *exo* double bond (*E*- and *Z*-configurations), or a chiral center (*S*- and *R*-configurations¹⁹) at position C-1 to control the spatial position of the amide group, which is one of the most important pharmacophores. We found compounds with the *S*-configuration at C-1 to be the most promising in terms of potency and selectivity for the MT₁ receptor. Furthermore, we suggested that the methyl orientation of the 6-methoxy group on the indan nucleus is very

* To whom correspondence should be addressed. Tel: +81-6-6300-6546. Fax: +81-6-6300-6306. E-mail: uchikawa_osamu@takeda.co.jp.

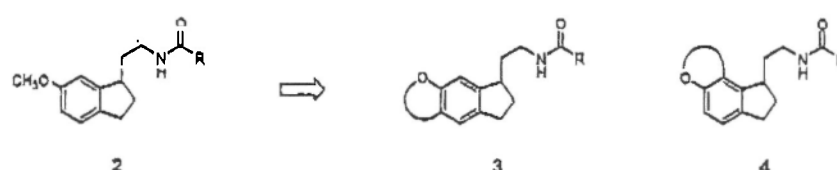
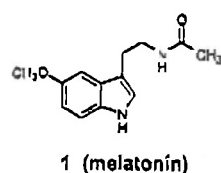
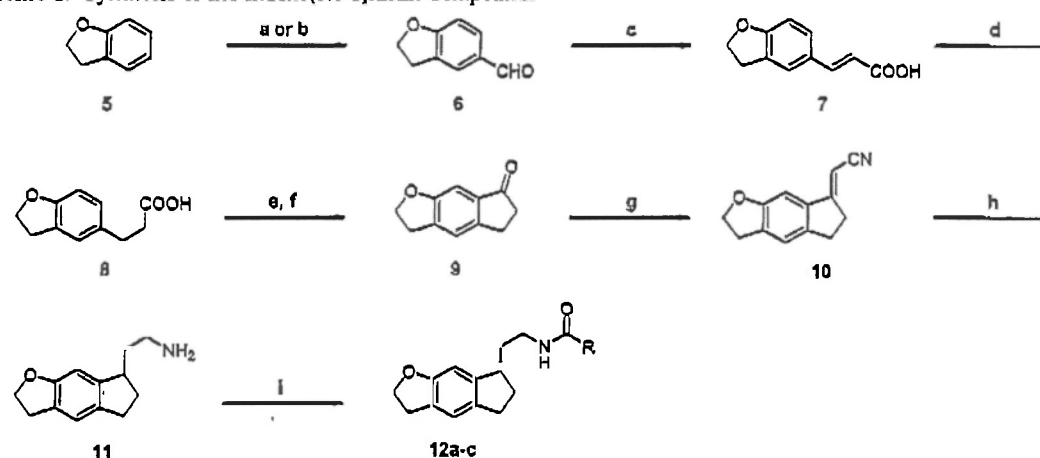


Figure 1. Progression from indan derivative 2 to tricyclic indan derivatives 3 and 4.

Scheme 1. Synthesis of the Indeno[5,6-*b*]furan Compounds^a



^a Reagents: (a) POCl₃, DMF; (b) TiCl₄, Cl₂CHCOCH₃, CH₂Cl₂; (c) Malonic acid, piperidine, pyridine; (d) H₂, Pd/C, AcOH; (e) SOCl₂; (f) AlCl₃, ClCH₂CH₂Cl; (g) (EtO)₂P(O)CH₂CN, NaH, THF; (h) H₂, Raney-Ni, NH₃-EtOH; (i) Ac₂O or RCOCl, NaOH, H₂O-THF.

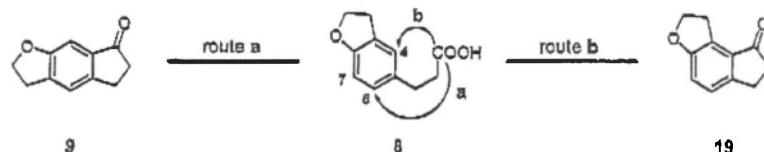


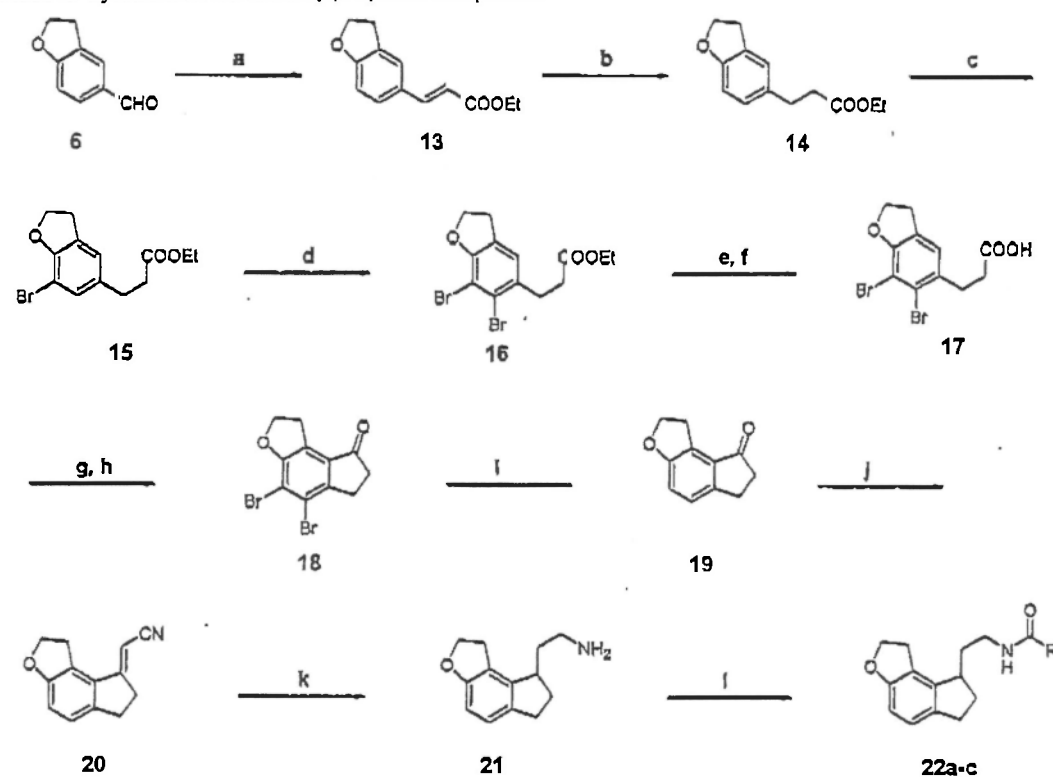
Figure 2. Construction of tricyclic indan analogues.

Important for the optimal orientation of the oxygen lone pairs with a view to obtaining the best binding at the receptor level. Thus, in this study, we explored the possibility of limiting the conformational flexibility of the methoxy group. It appeared to us desirable to design and synthesize conformationally restricted tricyclic analogues²⁰ as the indanic biosostere of melatonin, with the oxygen atom in the 6-position incorporated into a furan, 1,3-dioxolane, oxazole, pyran, morpholine, or 1,4-dioxane heterocyclic ring system.

This approach culminated in the discovery of indeno[5,4-*b*]furan analogues, which are not only metabolically stable but also very potent and selective for the MT₁ receptor, suggesting a superior *in vivo* activity and safety profile. We report in this paper the synthesis and biological activities of tricyclic analogues 3 and 4 as shown in Figure 1.

Chemistry

The 2,3-dihydrobenzofuran (5) was chosen as the starting material for the preparation of several indeno[5,4-*b*]furan derivatives (12a-c) as shown in Scheme 1. The formylation of 5 under Vilsmeier-Haack conditions²¹ gave the known aldehyde 6.²² A reaction with malonic acid followed by a hydrogenation reaction in the presence of 10% Pd on charcoal as a catalyst gave the carboxylic acid analogue 8. Friedel-Crafts cyclization²³ of 8 was accompanied by ring closure at C-6 of the benzofuran nucleus, to provide the linear tricyclic ketone 9 via the acyl chloride intermediate as shown in Figure 2 (route a). Compound 9 was subjected to a Horner-Emmons reaction²⁴ using diethyl cyanomethylphosphonate to give the intermediary unsaturated nitrile 10, which was converted to the amine 11 by hydrogenation

Scheme 2. Synthesis of the Indeno[5,4-*b*]furan Compounds^a

^a Reagents: (a) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{COOEt}$, NaH, THF. (b) H_2 , Pd/C, EtOH. (c) Br_2 , NaOAc, AcOH. (d) Br_2 , Fe, AcOH. (e) KOH, EtOH. (f) HCl. (g) SOCl_2 . (h) AlCl_3 , $\text{ClCH}_2\text{CH}_2\text{Cl}$. (i) H_2 , Pd/C, AcOH. (j) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{CN}$, NaH, THF. (k) H_2 , Raney-Ni, NH_3 -EtOH. (l) Ac_2O or RCOCl , NaOH, H_2O -THF.

over Raney-nickel. The *N*-acylated derivatives (22a-c) were prepared from 11 by treatment with the appropriate acyl chloride in the presence of sodium hydroxide as base and a biphasic medium according to the Schotten-Baumann procedure.²⁵

We turned our attention to the synthesis of angular indeno[5,4-*b*]furan derivatives (22a-c). Because the Friedel-Crafts reaction of 8 led to cyclization (route a) only at C-6 of the benzofuran nucleus as shown in Figure 2, we opted to explore another approach for the preparation of the angular tricyclic ketone 19 ring system (route b).

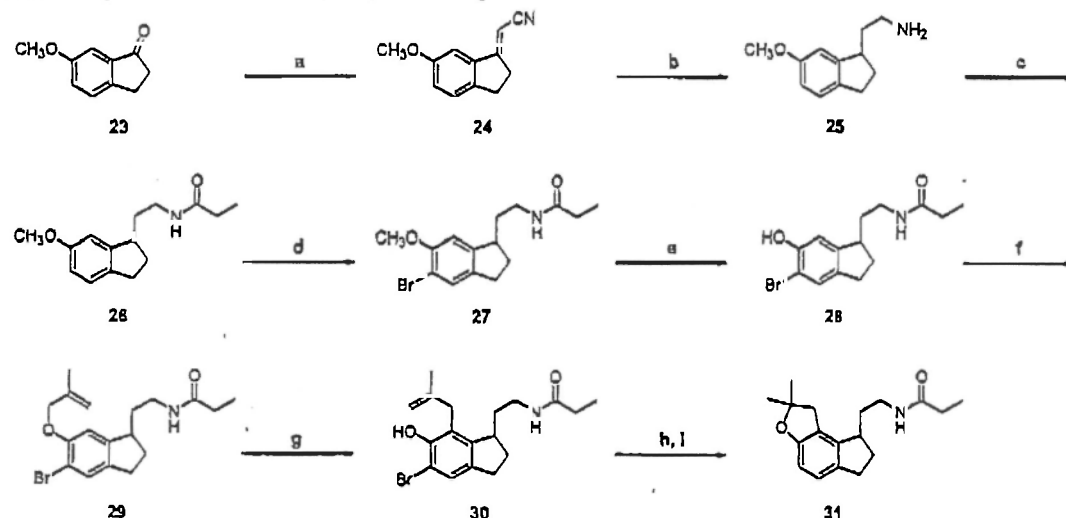
To avoid ring closure at C-6, we tried to block this position by introducing a bromine atom prior to cyclization. As outlined in Scheme 2, the aldehyde 6 was subjected to a Horner-Emmons reaction using triethyl phosphonoacetate, followed by hydrogenation in the presence of 10% Pd on charcoal to afford the objective, ester 14, which was brominated selectively in C-7 by bromine in acetic acid to give the monobrominated ester 15. The second bromination in C-6 was performed using 1.5 equiv of bromine in the presence of a catalytic amount of Fe.²⁶ The alkaline hydrolysis of 16 provided the corresponding carboxylic acid 17. As expected, the Friedel-Crafts cyclization of 17 occurred at C-4 leading to the angular tricyclic ketone 18. Debromination of 18 to 19 was readily achieved by hydrogenation in the presence of 10% Pd on charcoal. Compound 19 was transformed into the indeno[5,4-*b*]furan derivatives

(22a-c) in three steps involving the reaction of the ketone 19 with diethyl cyanomethylphosphonate, hydrogenation over Raney-Ni, and *N*-acylation.

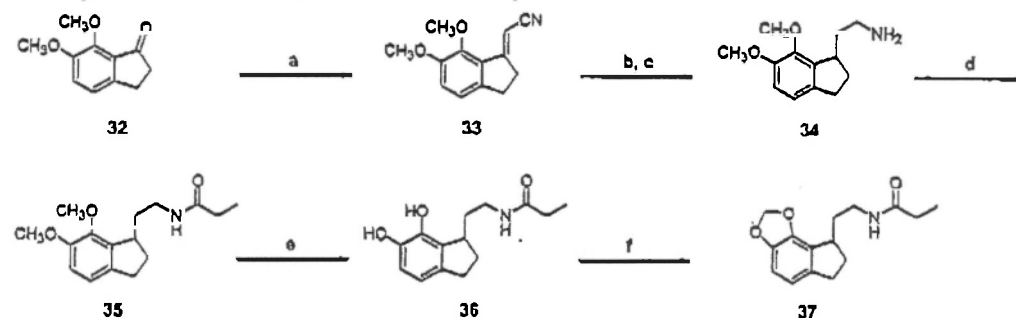
The 2,2-dimethyl-indeno[5,4-*b*]furan derivative (31) was prepared from commercially available 6-methoxyindanone (23), as shown in Scheme 3. Using the same conditions employed for the preparation of 22a-c, the ketone 23 was converted in three steps into the corresponding indan derivative 26. The bromide 27, obtained by bromination of 26, was allowed to react with boron tribromide to afford the alcohol 28, which was converted to the alkylated derivative 29 by reaction with 3-chloro-2-methylpropene. The Claisen rearrangement of 29 in *N,N*-diethylamine afforded 30. Acid-catalyzed cyclization of 30 with boron trifluoride diethyl etherate, followed by hydrogenation of the bromide, gave the desired product 2,2-dimethyl-indeno[5,4-*b*]furan (31).

The same strategy was used to prepare 37 bearing a 1,3-dioxolane ring condensed with the benzene ring (Scheme 4). The ketone 32²⁷ was converted in three steps into the corresponding indan derivative 35 by the same procedure employed in the preparation of 22a-c. Deprotection of the two methoxy groups of 35 with boron tribromide gave the diol 36, which was in turn treated with diiodomethane and sodium hydride in hexamethyl phosphoramide²⁸ to afford indeno[4,5-*d*][1,3]dioxole (37).

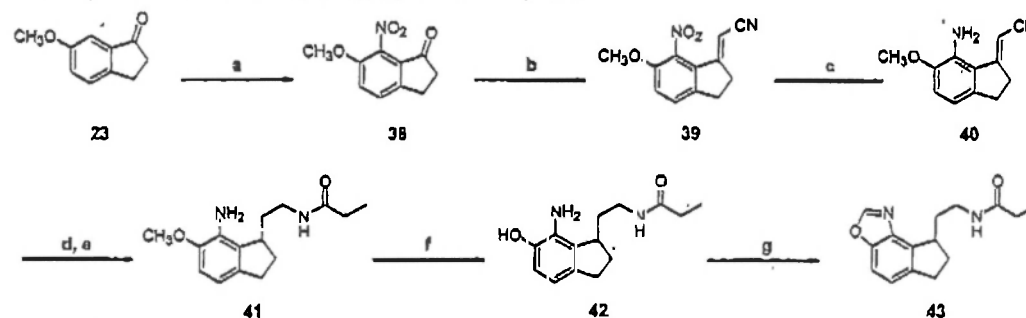
The 6-methoxyindanone (23) was also used for the preparation of the indeno[4,5-*d*][1,3]oxazole derivative (43) as shown in Scheme 5. Nitration of 23 with

Scheme 3. Synthesis of the Indeno[5,4-*b*]furan Compounds^a

^a Reagents: (a) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{CN}$, NaH, THF. (b) H_2 , Raney-Ni, $\text{NH}_3\text{-EtOH}$. (c) $\text{CH}_3\text{CH}_2\text{COCl}$, Et_3N , THF. (d) Br_2 , NaOAc, AcOH. (e) BBr_3 , CH_2Cl_2 . (f) 3-Chloro-2-methylpropane, NaH, DMF. (g) *N,N*-Diethylaniline, 200 °C. (h) $\text{BF}_3\text{-Et}_2\text{O}$, CH_2Cl_2 . (i) H_2 , Pd/C, EtOH.

Scheme 4. Synthesis of the Indeno[4,5-*d*][1,3]dioxole Compounds^a

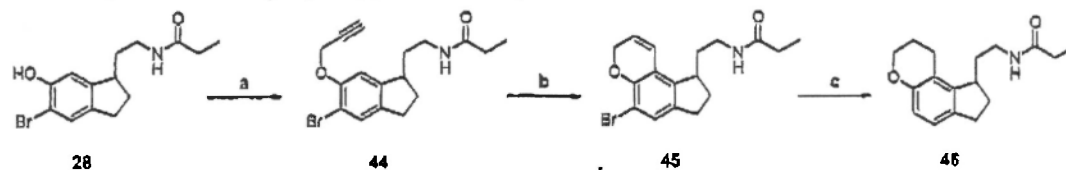
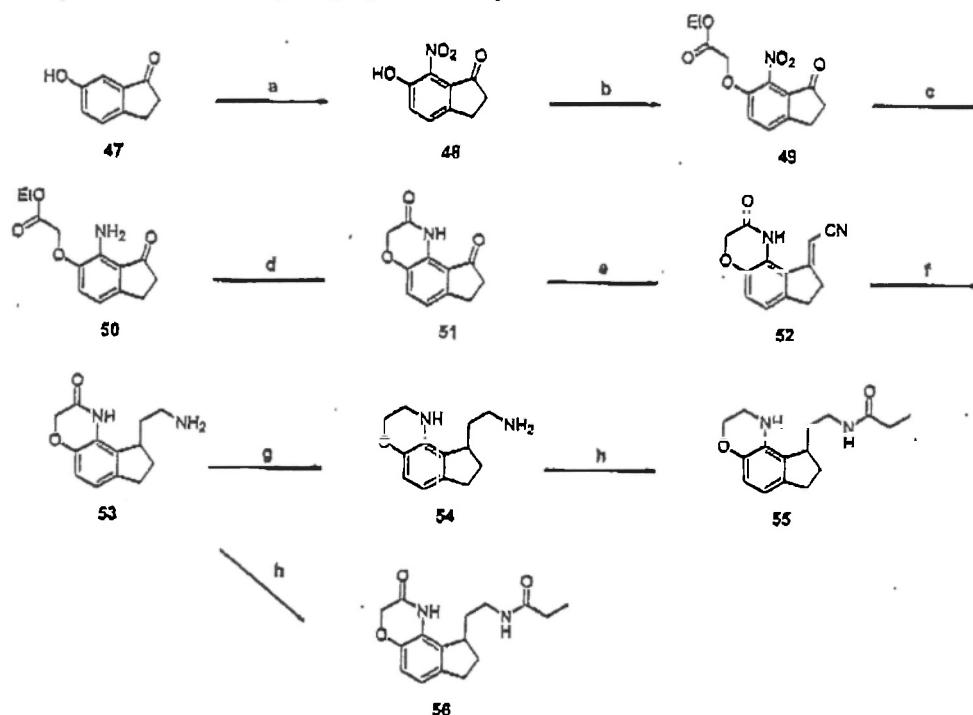
^a Reagents: (a) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{CN}$, NaH, THF. (b) H_2 , Raney-Co, $\text{NH}_3\text{-EtOH}$. (c) H_2 , Pd/C, EtOH. (d) $\text{CH}_3\text{CH}_2\text{COCl}$, Et_3N , THF. (e) BBr_3 , CH_2Cl_2 . (f) CH_2I_2 , NaH, HMPA.

Scheme 5. Synthesis of the Indeno[4,5-*d*][1,3]oxazole Compounds^a

^a Reagents: (a) KNO_3 , concentrated H_2SO_4 . (b) $(\text{EtO})_2\text{P}(\text{O})\text{CH}_2\text{CN}$, NaH, THF. (c) H_2 , Pd/C, EtOH. (d) H_2 , Raney-Ni, $\text{NH}_3\text{-EtOH}$. (e) $\text{CH}_3\text{CH}_2\text{COOH}$, WSC, HOBt, DMF. (f) BBr_3 , CH_2Cl_2 . (g) $\text{CH}(\text{OCH}_3)_2$, HCl-MeOH .

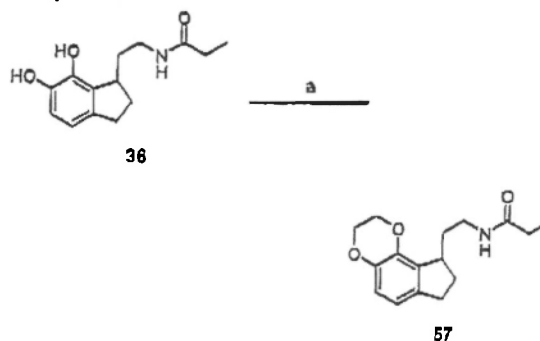
potassium nitrate in concentrated H_2SO_4 gave the nitro derivative 38. Compound 38 was subjected to a Horner–Emmons reaction to give the unsaturated nitrile 39, which was converted to the aniline 40 by catalytic hydrogenation. Hydrogenation over Raney-nickel of 40 and acylation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC)-mediated coupling of

propionic acid with the primary amine in the presence of 1-hydroxybenzotriazole monohydrate (HOBt) gave the indan 41. The indan 41 was converted to the phenol 42 by a reaction with boron tribromide, which was then transformed into the indeno[4,5-*d*][1,3]oxazole derivative (43) by heating in HCl-MeOH with methyl orthoformate.

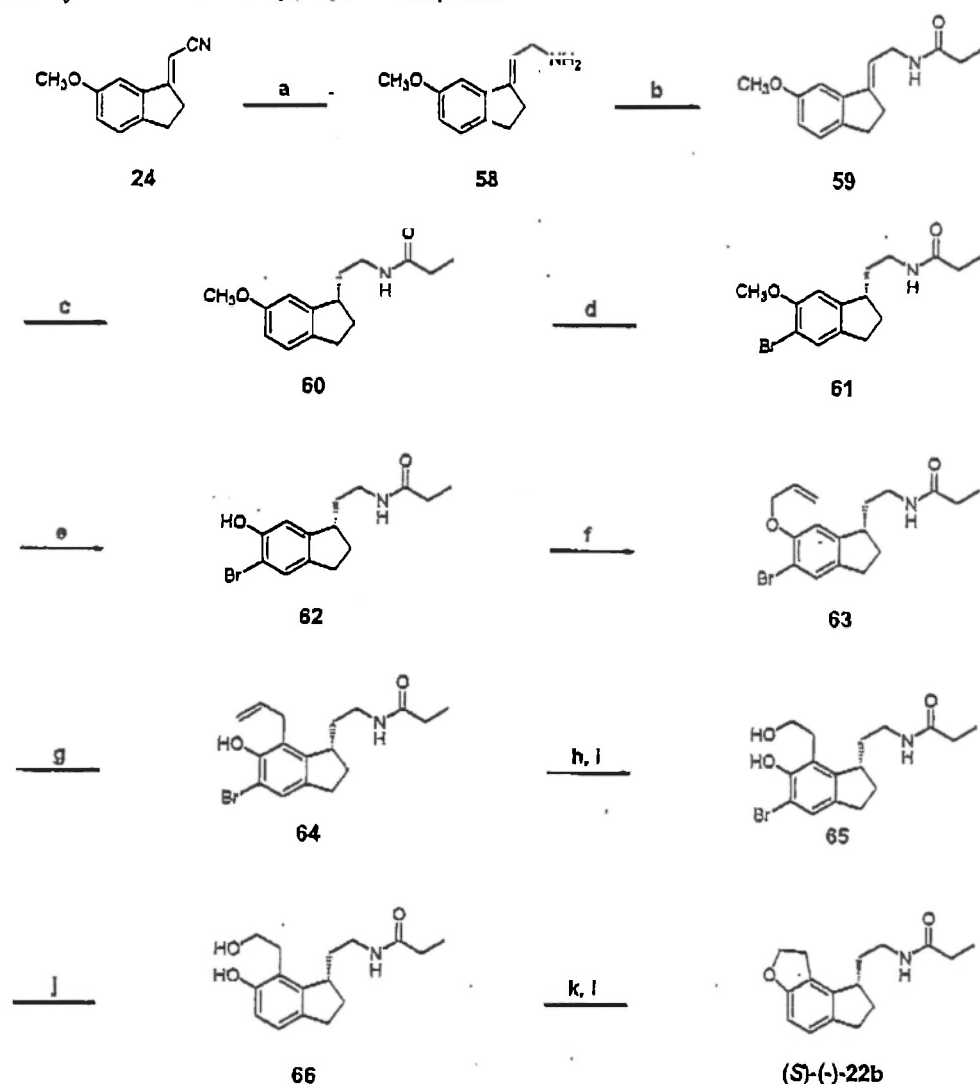
Scheme 6. Synthesis of the Cyclopenta[*f*]chromene Compounds^a^a Reagents: (a) Propargyl bromide, NaH, DMF. (b) Bromobenzene. (c) H₂, Pd/C, EtOH.Scheme 7. Synthesis of the Indeno[5,4-*b*][1,4]oxazine Compounds^a^a Reagents: (a) KNO₃, concentrated H₂SO₄. (b) NaH, BrCH₂COOEt, DMF. (c) H₂, Pd/C, EtOH. (d) tBuOK, toluene. (e) (EtO)₂P(O)CH₂CN, NaH, THF. (f) H₂, Raney-Ni, NH₃-EtOH. (g) LAH, THF. (h) CH₃CH₂COOH, WSC, HOBt, DMF.

The indan **28**, obtained in the preceding experiment, was treated with propargyl bromide and sodium hydride in *N,N*-dimethylformamide (DMF) to give the corresponding ether **44** (Scheme 6). Thermal intramolecular cyclization of the aryl propargyl ether **44** led to **45**, and catalytic reduction gave the angular cyclopenta[*f*]chromene derivative (**46**).

The amine **53** was the key material for the preparation of indeno[5,4-*b*][1,4]oxazine derivatives (Scheme 7). Nitration of 6-hydroxy-1-indanone (**47**) with potassium nitrate in concentrated H₂SO₄ followed by alkylation of the nitro compound **48** with ethyl bromoacetate gave the ester **49**, which was transformed into the key intermediate **53** in four straightforward steps, namely, reduction of the nitro group, cyclization of the obtained aminoester, condensation by the Horner–Emmons reaction, and reduction of the unsaturated nitrile **52**. After reduction of **53** with LiAlH₄, **54** was acylated to indeno[5,4-*b*][1,4]oxazine (**55**) using the WSC–HOBt procedure. The *N*-acylated derivative **56** was prepared directly from **53**.

Scheme 8. Synthesis of the Indeno[4,5-*b*][1,4]dioxine Compounds^a^a Reagents: (a) Br(CH₂)₂Br, CuO, K₂CO₃, DMF.

The indeno[4,5-*b*][1,4]dioxine (**57**) was prepared via the same diol **36** mentioned above by heating with dibromoethane, CuO, and K₂CO₃ in DMF. As with a related series of benzocycloalkene derivatives, the absolute configuration of the stereocenter had a significant

Scheme 9. Synthesis of the Indeno[5,4-*b*]furan Compounds^a

^a Reagents: (a) H_2 , Raney-Co, NH_3 -EtOH. (b) CH_3CH_2COCl , Et_3N , THF, 0 °C. (c) H_2 , $Ru(OAc)_2[(S)\text{-binap}]$, MeOH. (d) Br_2 , NaOAc, MeOH, 0 °C. (e) BBr_3 , CH_2Cl_2 , -20 °C. (f) $CH=CHCH_2Br$, NaH, DMF, 0 °C. (g) N,N -Diethylaniline, 200 °C. (h) O_3 , MeOH, -78 °C. (i) $NaBH_4$, MeOH, -78 °C. (j) H_2 , Pd/C, Et_3N , MeOH. (k) $MsCl$, pyridine, -10 to 0 °C. (l) Et_3N , EtOAc, reflux.

effect on the selectivity toward the MT_1 receptor. We therefore focused on the asymmetric synthesis of the (*S*)-isomer of **22b** according to the results of our previous study on indan derivatives. The key step was the asymmetric reduction of the *exo* double bond in the intermediate **59**. This reaction proceeded nicely under 9.1 MPa of hydrogen at 70 °C using the (*S*)-2,2'-bis-(diphenylphosphino)-1,1'-binaphthyl (binap)-Ru complex as a catalyst to give the enantiomerically pure **60** as we previously reported (Scheme 9).

Having solved the problem of asymmetric hydrogenation, we turned our attention to the construction of a furan ring condensed with the indan nucleus. Because of the low reactivity at C-7 of the indan ring, our attempts to introduce substituents using the Fries rearrangement, the Friedel-Crafts reaction, nitration,

or formylation for the construction of indeno[5,4-*b*]furan scaffolding were all unsuccessful. We finally attempted to introduce an allyl moiety into the C-7 position of the indan ring using a Claisen rearrangement and then cyclized it to dihydrobenzofuran. To block the more reactive C-5 position, bromination was performed. Subsequent boron tribromide treatment provided the 6-hydroxy indan **62**. Alkylation of **62** with allyl bromide gave **63**, which was then subjected to a Claisen rearrangement to afford **64**. Ozonolysis of the vinyl moiety followed by a reductive workup and deprotection of the C-5 bromide gave the diol **66**. The primary alcohol **66** was converted to mesylate and then cyclized to the desired (*S*)-(-)-**22b** by treatment with Et_3N in EtOAc at reflux.

Results and Discussion

MT₁ and MT₂ Receptor Binding Affinities. The affinities of the compounds for melatonin binding sites were evaluated by binding assays with 2-[¹²⁵I]iodo-melatonin radioligand using Chinese hamster ovary (CHO) cells expressing human melatonin receptor (MT₁ site) and Syrian hamster brain and peripheral organs (MT₂ site).

The affinities of compounds for these receptors are shown in Table 1. From the structure–affinity relationships, the position of the third ring condensed with the indan nucleus seems to be critical for binding affinity. This is supported by the fact that the angular tricyclic derivatives 22a–c showed a binding affinity for the MT₁ receptor that was ca. 3–4 orders of magnitude higher than that of the corresponding linear tricyclic derivatives 12a–c. These results were consistent with the observation that the methyl orientation of the 6-methoxy group on the indan nucleus was very important for the optimal orientation of the oxygen lone pairs as discussed previously. Regarding the length of the acyl side chain, although compounds 22a–c showed high affinity for the MT₁ receptors, there was a tendency for the affinity to improve with increasing chain length, the optimum group being the propionyl (22b). With respect to the MT₂ receptor, the increase of acyl chain length in the linear tricyclic series led to a decrease in the binding affinity whereas the corresponding angular tricyclic series gave the opposite results. As a consequence of these experiments, further optimization was pursued in the propionyl series. The introduction of dimethyl groups (31) into the furan ring was detrimental to the binding affinity for the MT₁ receptor. We therefore extensively modified the third ring part with substituents such as a 1,3-dioxolane (37), oxazole (43), pyran (46), morpholine (55 and 56), and 1,4-dioxane (57) heterocyclic ring. Replacement of the furan ring with a 1,3-dioxolane ring (37) preserved a moderate level of affinity for the MT₁ receptor, but further modification as seen with 43, 46 and 55–57 resulted in less affinity as compared with that of 22b. These results indicated that the angular unsubstituted dihydro furan ring condensed with the indan nucleus was the best structure. For a more rational explanation of the observed binding affinity, we carried out molecular orbital (MO) calculations of substrates 22b, 37, and 43 to compare the electron density on the oxygen atom. The calculations were made by the MNDO–PM₃ method with MOPAC version 6.00.²⁰ The net atomic charges of 22b and 37 were –0.19 and –0.20, respectively, and these compounds exhibited a similar high binding affinity. However, the value (–0.11) of 43 is worth noting, suggesting that the parameter accounts for the weak potency.

To investigate the stereochemical requirements, the two enantiomers, (S)-(–)-22b and (R)-(+)-22b, of compound 22b were prepared and examined for binding affinity. As expected, significant differences were observed and (S)-(–)-22b was more than 500 times as potent as (R)-(+)-22b for the MT₁ receptor, confirming the favorable influence of the S-configuration. Compound (S)-(–)-22b had the most impressive binding profile for the MT₁ (*K*_i = 13.8 pM) receptor. (S)-(–)-22b showed very low affinity for the MT₂ receptor with a *K*_i

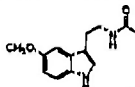
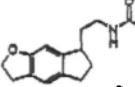
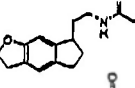
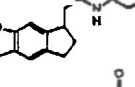
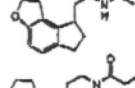
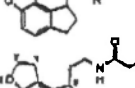
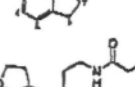
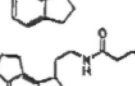
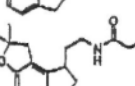
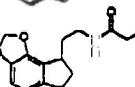
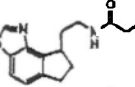
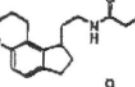
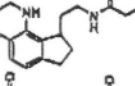
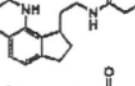
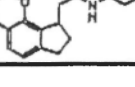
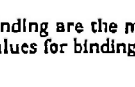
value of 2.6 μM, which was 190 000-fold the value for the human MT₁ subtype. These results indicate that (S)-(–)-22b is a potent and selective MT₁ agonist and warrants further pharmacological study.

Adenosine Cyclic 3',5'-Phosphate (cAMP) Production in the Rat Pituitary. This experiment was based on the method of Vanccek and Vollrath³⁰ with minor modifications. The stimulation of 9 day old rat pituitary with 1 μM forskolin raised the cAMP content to 10 times the basal level. Melatonin inhibited forskolin-stimulated cAMP production in a concentration-dependent manner, the effect of which was maximal at 1 nM reaching about 50–65% inhibition. (S)-(–)-22b, like melatonin, inhibited cAMP production, indicating an agonistic effect on the melatonin receptor in the pituitary, although its activity was slightly weaker than that of melatonin, as shown by the IC₅₀ values in Table 2.

Effects of (S)-(–)-22b and Melatonin on Sleep and Wakefulness in Freely Moving Cats. The sleep-promoting effects were studied in freely moving cats. Administration of melatonin (1 mg/kg, po) reduced wakefulness and increased slow wave sleep (SWS) and rapid eye movement (REM) sleep (analysis of variance (ANOVA), both *p* < 0.01). However, the duration of sleep-inducing action was not very long and significant effects were observed only for 2 h after the administration, although the effect on REM was not significant (Figure 3). (S)-(–)-22b (0.1 mg/kg, po) had potent sleep-promoting activity, as indicated by the significant decrease in wakefulness and significant increases in SWS and REM sleep (ANOVA, *p* < 0.05 or *p* < 0.01). The effects lasted for about 6 h after the administration (Figure 4). Thus, the sleep-inducing action of (S)-(–)-22b and the duration of the effect were much greater than for melatonin.

Docking Study. To explain the potency of (S)-(–)-22b, we performed a docking experiment using a model of the human melatonin receptor (MT₁) constructed from the crystal structure of bovine rhodopsin.³¹ Details of the model are given in the Experimental Section. Figure 5 shows the most reasonable mode of binding of (S)-(–)-22b in the ligand binding pocket. In this model, the oxygen atom in the furan ring forms a hydrogen bond with His195 and the adjacent carbon atom interacts with Val192. Both residues are located in transmembrane segment V and are suggested to be concerned with ligand recognition.³² The orientation of the oxygen lone pairs incorporated into the furan ring would be important as can be seen from the data on the binding affinity of 12b and (S)-(–)-22b. Actually, the orientation of the oxygen lone pairs of 12b as compared to (S)-(–)-22b would be unfavorable for a strong hydrogen-bonding interaction, though the furan ring of 12b can be accommodated around His195 (data not shown). Fixing the orientation of the methoxy moiety of melatonin by its incorporation into a furan ring seems to play a main role in reinforcing the interaction. The propanamide side chain is favorably surrounded by Val261 and Pro265, both in transmembrane segment VI and is simultaneously hydrogen-bonded to Tyr175 and Ser182 in the second extracellular loop, which is suggested to be important for ligand binding.³³ The interaction with Pro265, which is not available for melatonin, would

Table 1. Binding Affinity and Physical Properties of Tricyclic Analogues

compound		receptor binding (K^a , nM)		mp ($^{\circ}\text{C}$)	formula	anal. ^d
		MT ₁ ^b	MT ₂ ^c			
melatonin		0.0823 $\pm$ 0.0021	27.6 $\pm$ 0.3			
12a		483 $\pm$ 131	237 $\pm$ 69	127-128	C ₁₉ H ₁₉ NO ₂	C, H, N
12b		255 $\pm$ 45	255 $\pm$ 101	125-126	C ₁₆ H ₂₁ NO ₂	C, H, N
12c		240 $\pm$ 35	492 $\pm$ 199	120-121	C ₁₇ H ₂₃ NO ₂	C, H, N
22a		0.126 $\pm$ 0.035	5960 $\pm$ 1330	78-79	C ₁₉ H ₁₉ NO ₂	C, H, N
22b		0.0174 $\pm$ 0.0041	1250 $\pm$ 191	102-104	C ₁₆ H ₂₁ NO ₂	C, H, N
(S)-(-)-22b		0.0138 $\pm$ 0.0006	2600 $\pm$ 190	113-115	C ₁₆ H ₂₁ NO ₂	C, H, N
(R)-(+)-22b		7.31 $\pm$ 2.86	2270 $\pm$ 656	113-115	C ₁₆ H ₂₁ NO ₂	C, H, N
22c		0.0214 $\pm$ 0.0050	1070 $\pm$ 271	55-57	C ₁₇ H ₂₃ NO ₂	C, H, N
31		0.422 $\pm$ 0.070	4790 $\pm$ 1860	69-72	C ₁₈ H ₂₁ NO ₂	C, H, N
37		0.0241 $\pm$ 0.0059	5830 $\pm$ 1740	102-104	C ₁₅ H ₁₉ NO ₂	C, H, N
43		3.29 $\pm$ 0.63	52.8 $\pm$ 18.0	81-84	C ₁₇ H ₁₉ N ₃ O ₂	C, H, N
46		0.242 $\pm$ 0.035	6220 $\pm$ 1920	85-88	C ₁₇ H ₂₃ NO ₂	C, H, N
55		15.3 $\pm$ 5.10	> 10000	80-83	C ₁₆ H ₂₁ N ₃ O ₂	C, H, N
56		326 $\pm$ 35	2560 $\pm$ 854	216-219	C ₁₈ H ₂₃ N ₃ O ₂	C, H, N
57		0.423 $\pm$ 0.088	5390 $\pm$ 165	120-122	C ₁₈ H ₂₁ NO ₂	C, H, N

^a K_i values for binding are the means of three experiments. ^b Values for binding affinity in CHO cells expressing human melatonin (MT₁) receptor. ^c Values for binding affinity in syrian hamster brain and peripheral organs. ^d Analytical results are within $\pm 0.4\%$ of the theoretical value.

Table 2. Effects of (S)-(-)-22b on cAMP Production in the Neonatal Rat Pituitary^a

compd	IC ₅₀ (pM)	K _i (pM)	cAMP/human
	cAMP production	human (MT ₁)	
(S)-(-)-22b	20.8	13.8	1.51
melatonin	79.8	82.3	0.97

^aIC₅₀ values for cAMP production were calculated from the mean data of two experiments each done in triplicate. K_i values for binding are the means of three experiments.

reinforce the binding of (S)-(-)-22b in this moiety. The receptor has many hydrophobic residues at the bottom of the pocket. The hydrophobic interaction at this site caused by replacing the indole nitrogen atom in melatonin with a methylene unit would contribute to the high affinity of (S)-(-)-22b.

Conclusion

We synthesized a variety of tricyclic indan analogues to maximize affinity for the MT₁ receptor in consideration of the interaction between two valuable pharmacophores and MT₁ receptors based on the results of our previous study. The angular indeno[5,4-*b*]furan structure serves as an effective, conformationally restricted bioisostere of melatonin, and the orientation controlled by the furan ring and the propanamide side chain

exemplified by (S)-(-)-22b most likely represents the active conformation during melatonin receptor activation.

(S)-(-)-22b showed remarkably high affinity for the human MT₁ receptor with a K_i value of 13.8 pM, whereas it showed very low affinity for the hamster MT₂ receptor with a K_i value of just 2.6 nM. The affinities for MT₁ and MT₂ receptors were 6-fold higher and 94-fold lower than those of melatonin, respectively. (S)-(-)-22b inhibited forskolin-stimulated cAMP production in the neonatal rat pituitary with an IC₅₀ of 20.8 pM, indicating that this compound is a MT₁ agonist. The pharmacological effects of (S)-(-)-22b were studied in experimental animals. (S)-(-)-22b (0.1 mg/kg, po) showed a sleep-promoting action in freely moving cats, as demonstrated by a decrease in wakefulness and increases in SWS and REM sleep, which lasted for 6 h after the administration. These results indicate that (S)-(-)-22b is a potent and highly selective agonist for the MT₁ melatonin receptor, which is expected to be a useful therapeutic for sleep and/or circadian rhythm disorders, and a valuable tool in pharmacological investigations of the structure-activity relationships of melatonergic agents. (S)-(-)-22b also showed high affinity for the MT₂ receptor with a K_i value of 0.045 ± 0.018 nM, which was 4-fold higher than melatonin (K_i = 0.195 ± 0.051

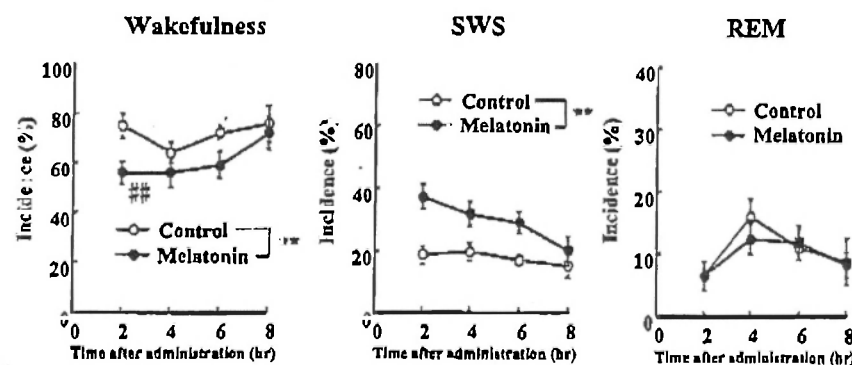


Figure 3. Effects of melatonin on sleep and wakefulness in freely moving cats. The experiment was based on the crossover design. Eight cats were treated with melatonin (1 mg/kg, po) or vehicle. Each value shows the mean percentage at each stage during each block of 2 h. ***p* < 0.01, main group effect by ANOVA; **p* < 0.01, as compared with the vehicle control (paired *t*-test with Holm's correction).

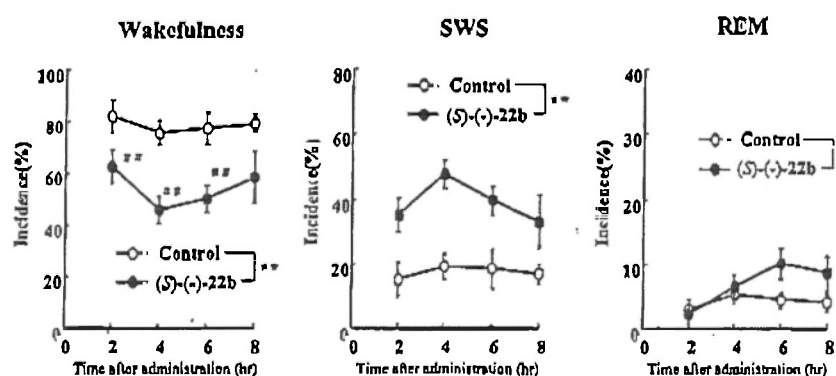


Figure 4. Effects of (S)-(-)-22b on sleep and wakefulness in freely moving cats. The experiment was based on the crossover design. Eight cats were treated with (S)-(-)-22b (0.1 mg/kg, po) or vehicle. Each value shows the mean percentage at each stage during each block of 2 h. **p* < 0.05 and ***p* < 0.01, main group effect by ANOVA; **p* < 0.01, as compared with the vehicle control (paired *t*-test with Holm's correction).

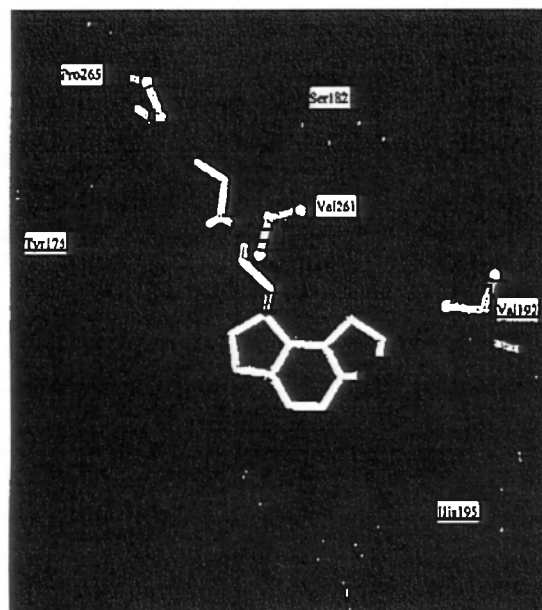


Figure 5. Schematic model for the binding of (S)-(-)-22b to the MT₁ receptor.

nM). This affinity for the MT₂ receptor may be related to the pharmacological effect of (S)-(-)-22b. Furthermore, we have developed a practical method for the stereospecific synthesis of optically active (S)-(-)-22b by transforming the indan ring into the angular tricyclic ring via catalytic asymmetric hydrogenation with the (S)-binap-Ru complex. (S)-(-)-22b is a promising drug candidate for the treatment of primary insomnia and circadian rhythm disorders and is now under clinical evaluation.

Experimental Section

A. Chemistry. Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were determined with a JASCO DIP-370 digital polarimeter. Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on a Varian Gemini-200 spectrometer in the solvent indicated. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations are used as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Coupling constants (*J* values) are given in hertz (Hz). Thin-layer chromatography (TLC) was performed on silica gel 60F₂₅₄ (Merck), and column chromatography was carried out on a silica gel column (Kieselgel 60, 0.063–0.200 mm, Merck). Evaporation was carried out in vacuo on a rotary evaporator. (E)-2-(6-Methoxy-2,3-dihydro-1*H*-inden-1-ylidene)acetonitrile (24), 6-methoxy-2,3-dihydro-1*H*-inden-1-ethanamine (25), *N*-[2-(6-methoxy-2,3-dihydro-1*H*-inden-1-yl)ethyl]propanamide (26), 2-(6-methoxy-2,3-dihydro-1*H*-inden-1-ylidene)ethylaniline (58), *N*-[2-(6-methoxy-2,3-dihydro-1*H*-inden-1-ylidene)ethyl]propanamide (59), and (S)-*N*-[2-(6-methoxy-2,3-dihydro-1*H*-inden-1-yl)ethyl]propanamide (60) were synthesized according to a process as described in the previous paper.

2,3-Dihydro-1-benzofuran-5-carbaldehyde (6).²² Method A. Phosphorus oxychloride (31.0 mL, 0.333 mol) was slowly added to a solution of DMF (28.4 mL, 0.386 mol) while the temperature was maintained below 50 °C. Then, 2,3-dihydrobenzofuran (5) (20.0 g, 0.166 mol) was added dropwise to this reaction mixture at below 50 °C, over a period of 10 min.

The resulting mixture was warmed and stirred at 80–85 °C for 12 h. The reaction mixture was then poured into ice-water and carefully quenched with a solution of sodium hydroxide. The organic material was separated, and the aqueous layer was extracted with ethyl acetate. It was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (8:2) as eluent followed by distillation to yield 21.2 g (86%) of 6; bp 93–96 °C/0.2–0.3 mm Hg. ¹H NMR (CDCl₃): δ 3.28 (t, 2H, *J* = 8.8), 4.70 (t, 2H, *J* = 8.8), 6.88 (d, 1H, *J* = 8.4), 7.67 (dd, 1H, *J* = 1.0, 8.4), 7.75 (d, 1H, *J* = 1.0), 9.83 (s, 1H). Anal. (C₁₀H₈O₂) C, H.

Method B. Titanium(IV) chloride (28 mL) was added to an ice-cooled solution of 5 (10.0 g, 83.2 mmol) and dichloromethyl methyl ether (11.3 mL, 0.125 mmol) in dichloromethane (100 mL). After 1 h, the ice-bath was removed and the dark mixture was quenched by the addition of ice-water. The aqueous layer was discarded, and the organic phase was washed with water and brine and then dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (1:1) as eluent to yield 11.4 g (92%) of 6 as an oil.

3-(2,3-Dihydro-1-benzofuran-5-yl)-2-propenoic Acid (7). A mixture of 6 (4.99 g, 33.7 mmol) and malonic acid (5.27 g, 50.6 mmol) in pyridine (30 mL) containing piperidine (0.192 g, 2.25 mmol) was heated at 100 °C for 4 h. The cooled reaction mixture was concentrated, and the residue was poured into water. It was acidified with 6 N HCl and extracted with methanol/chloroform (1:9). The extract was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with methanol/chloroform (5:95) as eluent to yield 3.98 g (62%) of 7; mp 173–176 °C (from ethyl acetate). ¹H NMR (CDCl₃): δ 3.24 (t, 2H, *J* = 8.8), 4.64 (t, 2H, *J* = 8.8), 6.28 (d, 1H, *J* = 15.9), 6.55 (d, 1H, *J* = 8.2), 7.34 (d, 1H, *J* = 8.2), 7.44 (s, 1H), 7.74 (d, 1H, *J* = 15.9), hidden (1H). Anal. (C₁₁H₁₀O₃) C, H.

3-(2,3-Dihydro-1-benzofuran-5-yl)propanoic Acid (8). A solution of 7 (3.75 g, 19.7 mmol) in acetic acid (50 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (0.5 g, containing 50% water) in a hydrogen atmosphere at room temperature for 1 h. After absorption of the theoretical volume of hydrogen, the catalyst was removed by filtration. The filtrate was concentrated and washed with hexane to yield 3.41 g (90%) of 8. The analytical sample was obtained by recrystallization from a mixture of ethyl acetate and hexane; mp 95–98 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 2.63 (t, 2H, *J* = 7.5), 2.89 (t, 2H, *J* = 7.5), 3.18 (t, 2H, *J* = 8.8), 4.55 (t, 2H, *J* = 8.8), 6.70 (d, 1H, *J* = 8.1), 6.94 (d, 1H, *J* = 8.1), 7.04 (s, 1H), hidden (1H). Anal. (C₁₁H₁₂O₃) C, H.

2,3,5,6-Tetrahydro-7*H*-indeno[5,6-*b*]furan-7-one (9). Compound 8 (5.84 g, 30.4 mmol) was added to thionyl chloride (6.64 mL, 19.9 mol) followed by stirring at 75 °C for 40 min. After excess reagent was removed, the residue was dissolved in 1,2-dichloroethane (10 mL). One-third of the solution of acid chloride in 1,2-dichloroethane was added to an ice-cooled suspension of anhydrous aluminum chloride (1.49 g, 11.2 mmol) in 1,2-dichloroethane (150 mL). After the mixture was stirred for 15 min, anhydrous aluminum chloride (2.97 g, 22.3 mmol) and the rest of the solution of acid chloride were added, followed by stirring at room temperature further for 15 min. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with 1 N HCl, 1 N NaOH, and brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (7:3) as eluent and recrystallized from a mixture of ethyl acetate and isopropyl ether to yield 4.09 g (77%) of 9; mp 110–111 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 2.66–2.74 (m, 2H), 2.99–3.07 (m, 2H), 3.27 (t, 2H, *J* = 8.6), 4.64 (t, 2H, *J* = 8.6), 7.07 (s, 1H), 7.27 (s, 1H).

2-(2,3,5,6-Tetrahydro-7*H*-indeno[5,6-*b*]furan-7-ylidene)acetonitrile (10). A 60% suspension of sodium hydride in

mineral oil (0.744 g, 18.6 mmol) was added to a solution of diethyl cyanomethylphosphonate (3.30 g, 18.6 mmol) in tetrahydrofuran (40 mL). The mixture was stirred at room temperature for 30 min and was added dropwise at room temperature to a stirred mixture of 9 (2.95 g, 16.9 mmol) in tetrahydrofuran (20 mL). After the addition was complete, the reaction mixture was stirred at room temperature a further 2 h. Water was added, and the mixture was extracted with ethyl acetate. The extract was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (1:1) as eluent and recrystallized from a mixture of ethyl acetate and hexane to yield 2.20 g (66%) of 10; mp 141–142 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 2.97–3.04 (m, 2H), 3.06–3.17 (m, 2H), 3.22 (t, 2H, *J* = 8.0), 4.61 (t, 2H, *J* = 8.6), 5.50–5.52 (m, 1H), 6.84 (s, 1H), 7.16 (s, 1H).

2-(3,5,6,7-Tetrahydro-2*H*-indeno[5,6-*b*]furan-7-yl)ethylamine (11). A solution of 10 (2.15 g, 10.9 mmol) in ethanol (50 mL) saturated with NH₃ gas was subjected to hydrogenation in the presence of Raney-nickel (2.0 g washed with ethanol two times before use) in a hydrogen atmosphere (480 kPa) at 40 °C for 5 h. After hydrogen absorption ceased, the catalyst was removed by filtration. The filtrate was concentrated to yield 2.10 g (95%) of 11 as an oil, which was used directly in the next step. ¹H NMR (CDCl₃): δ 1.39 (br s, 2H), 1.47–1.77 (m, 2H), 1.86–2.03 (m, 1H), 2.20–2.38 (m, 1H), 2.65–2.94 (m, 4H), 3.02–3.18 (m, 1H), 3.15 (t, 2H, *J* = 8.6), 4.55 (t, 2H, *J* = 8.6), 6.63 (s, 1H), 7.02 (s, 1H).

***N*-(2-(3,5,6,7-Tetrahydro-2*H*-indeno[5,6-*b*]furan-7-yl)ethyl)acetamide (12a).** Acetic anhydride (0.168 mL, 1.78 mmol) was added all at once to a vigorously stirred solution of 11 (0.301 g, 1.48 mmol) and aqueous 1 N NaOH (2.5 mL) in tetrahydrofuran (3 mL) at room temperature. The mixture was stirred for 30 min at room temperature, and ethyl acetate and water were added. The organic layer was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and hexane to yield 0.280 g (77%) of 12a; mp 127–128 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.48–1.80 (m, 2H), 1.94–2.10 (m, 1H), 1.97 (s, 3H), 2.23–2.41 (m, 1H), 2.66–2.92 (m, 2H), 3.00–3.19 (m, 3H), 3.31–3.42 (m, 2H), 4.55 (t, 2H, *J* = 8.6), 5.52 (br s, 1H), 6.61 (s, 1H), 7.02 (s, 1H). Anal. (C₁₅H₁₉NO₂) C, H, N.

***N*-(2-(3,5,6,7-Tetrahydro-2*H*-indeno[5,6-*b*]furan-7-yl)ethyl)propionamide (12b).** Compound 12b was prepared in 71% yield from propionyl chloride and 11 by a method similar to that described for 12a; mp 125–126 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.15 (t, 3H, *J* = 7.6), 1.49–1.80 (m, 2H), 1.93–2.09 (m, 1H), 2.15–2.41 (m, 3H), 2.65–2.92 (m, 2H), 3.00–3.19 (m, 3H), 3.33–3.43 (m, 2H), 4.55 (t, 2H, *J* = 8.6), 5.46 (br s, 1H), 6.61 (s, 1H), 7.02 (s, 1H). Anal. (C₁₆H₂₁NO₂) C, H, N.

***N*-(2-(3,5,6,7-Tetrahydro-2*H*-indeno[5,6-*b*]furan-7-yl)ethyl)butanamide (12c).** Compound 12c was prepared in 72% yield from butyryl chloride and 11 by a method similar to that described for 12a; mp 120–121 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 0.94 (t, 3H, *J* = 7.2), 1.49–1.81 (m, 4H), 1.94–2.18 (m, 3H), 2.23–2.40 (m, 1H), 2.65–2.92 (m, 2H), 2.99–3.19 (m, 3H), 3.12–3.43 (m, 2H), 4.55 (t, 2H, *J* = 8.6), 5.45 (br s, 1H), 6.61 (s, 1H), 7.02 (s, 1H). Anal. (C₁₇H₂₃NO₂) C, H, N.

Ethyl (E)-3-(2,3-Dihydro-1-benzofuran-5-yl)-2-propenoate (13). A 60% suspension of sodium hydride in mineral oil (3.39 g, 84.6 mmol) was added to a solution of triethyl phosphonoacetate (19.0 g, 84.6 mmol) in tetrahydrofuran (150 mL). The mixture was stirred at room temperature for 20 min and was added dropwise at room temperature to a stirred mixture of 6 (11.4 g, 76.9 mmol) in tetrahydrofuran (15 mL). After the addition was complete, the reaction mixture was stirred at room temperature a further 1 h. Water was added, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate. The extract was washed with brine and dried over anhydrous magnesium sulfate. The

filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (from 95:5 to 9:1) as eluent to yield 14.7 g (88%) of 13 as an oil. ¹H NMR (CDCl₃): δ 1.33 (t, 3H, *J* = 7.2), 3.23 (t, 2H, *J* = 8.8), 4.25 (q, 2H, *J* = 7.2), 4.63 (t, 2H, *J* = 8.8), 6.28 (d, 1H, *J* = 16.0), 6.79 (d, 1H, *J* = 8.4), 7.31 (d, 1H, *J* = 8.4), 7.41 (s, 1H), 7.64 (d, 1H, *J* = 16.0).

Ethyl 3-(2,3-Dihydro-1-benzofuran-5-yl)propanoate (14). A solution of 13 (14.7 g, 66.7 mmol) in ethanol (150 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (1 g, containing 50% water) in a hydrogen atmosphere at room temperature for 2 h. After the theoretical volume of hydrogen was absorbed, the catalyst was removed by filtration. The filtrate was concentrated to yield 14.6 g (89%) of 14 as an oil, which was used directly in the next step. ¹H NMR (CDCl₃): δ 1.24 (t, 3H, *J* = 7.2), 2.57 (t, 2H, *J* = 7.8), 2.88 (t, 2H, *J* = 7.8), 3.18 (t, 2H, *J* = 8.0), 4.13 (q, 2H, *J* = 7.2), 4.55 (t, 2H, *J* = 8.0), 6.70 (d, 1H, *J* = 8.2), 6.94 (d, 1H, *J* = 8.2), 7.05 (s, 1H).

Ethyl 3-(7-Bromo-2,3-dihydro-1-benzofuran-5-yl)propanoate (15). A solution of 14 (14.5 g, 65.8 mmol) and sodium acetate (5.94 g, 72.4 mmol) in acetic acid (150 mL) was stirred at room temperature, and bromine (10.5 g, 65.8 mmol) was added dropwise over a period of 15 min. After 1 h at room temperature, the mixture was evaporated under reduced pressure. The residual oil was taken up in ethyl acetate and washed successively with aqueous 5% NaHSO₃, saturated NaHCO₃, and H₂O before it was dried over anhydrous magnesium sulfate. The filtrate was concentrated to yield 19.2 g (97%) of 15 as an oil, which was used directly in the next step. ¹H NMR (CDCl₃): δ 1.25 (t, 3H, *J* = 7.2), 2.57 (t, 2H, *J* = 7.6), 2.85 (t, 2H, *J* = 7.6), 3.28 (t, 2H, *J* = 8.8), 4.13 (q, 2H, *J* = 7.2), 4.65 (t, 2H, *J* = 8.8), 6.97 (s, 1H), 7.11 (s, 1H).

Ethyl 3-(6,7-Dibromo-2,3-dihydro-1-benzofuran-5-yl)propanoate (16). A suspension of 15 (1.00 g, 3.34 mmol) and iron (10 mg) in acetic acid (10 mL) was stirred at room temperature, and bromine (0.801 g, 5.01 mmol) was added dropwise over a period of 15 min. After the mixture was stirred for 5 h at 50 °C, iron was removed by filtration and concentrated under reduced pressure. The residual oil was taken up in ethyl acetate, washed successively with aqueous 5% NaHSO₃, saturated NaHCO₃, and H₂O, and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate/hexane (1:3) as eluent to yield 670 mg (53%) of 16; mp 42–43 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.25 (t, 3H, *J* = 7.3), 2.60 (t, 2H, *J* = 7.7), 3.07 (t, 2H, *J* = 7.7), 3.27 (t, 2H, *J* = 8.8), 4.14 (q, 2H, *J* = 7.3), 4.68 (t, 2H, *J* = 8.8), 7.06 (s, 1H).

3-(6,7-Dibromo-2,3-dihydro-1-benzofuran-5-yl)propanoic Acid (17). Aqueous KOH (0.138 g, 2.46 mmol) was added slowly to an ice-cooled solution of 16 (0.620 g, 1.64 mmol) in EtOH (10 mL). The solution was heated at 90 °C for 1 h. The mixture was poured into 5 N HCl and extracted with ethyl acetate. The extract was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and hexane to yield 0.53 g (93%) of 17; mp 117–118 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 2.67 (t, 2H, *J* = 7.5), 3.08 (t, 2H, *J* = 7.5), 3.27 (t, 2H, *J* = 8.8), 4.68 (t, 2H, *J* = 8.8), 7.07 (s, 1H), hidden (1H).

4,5-Dibromo-1,2,6,7-tetrahydro-8*H*-indeno[5,4-*b*]furan-8-one (18). Compound 18 was prepared in 88% yield from 17 by a method similar to that described for 9; mp 224–226 °C (from chloroform/isopropyl ether). ¹H NMR (CDCl₃): δ 2.72 (t, 2H, *J* = 5.9), 3.05 (t, 2H, *J* = 5.9), 3.55 (t, 2H, *J* = 9.0), 4.79 (t, 2H, *J* = 9.0).

1,2,6,7-Tetrahydro-8*H*-indeno[5,4-*b*]furan-8-one (19). A solution of 18 (0.349 g, 1.05 mmol) in AcOH (150 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (0.45 g, containing 50% water) in a hydrogen atmosphere at room temperature for 1 h. After the theoretical volume of hydrogen was absorbed, the catalyst was removed by filtration. The filtrate was concentrated, and the residue

was dissolved in ethyl acetate. This solution was washed with water and saturated aqueous NaHCO_3 and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography to yield 0.163 g (89%) of 19; mp 133–134 °C (from ethyl acetate/hexane). ^1H NMR (CDCl_3): δ 2.68 (t, 2H, $J = 5.9$), 3.08 (t, 2H, $J = 5.9$), 3.47 (t, 2H, $J = 8.8$), 4.65 (t, 2H, $J = 8.8$), 7.01 (d, 1H, $J = 8.1$), 7.21 (d, 1H, $J = 8.1$). Anal. ($\text{C}_{11}\text{H}_{10}\text{O}_2$) C, H.

2-(1,2,6,7-Tetrahydro-8H-indeno[5,4-b]furan-8-ylidene)acetonitrile (20). Compound 20 was prepared in 60% yield from 19 by a method similar to that described for 10; mp 149–151 °C (from methanol). ^1H NMR (CDCl_3): δ 3.00–3.20 (m, 4H), 3.31 (t, 2H, $J = 8.8$), 4.67 (t, 2H, $J = 8.8$), 5.45 (t, 1H, $J = 2.4$), 6.86 (d, 1H, $J = 8.1$), 7.11 (d, 1H, $J = 8.1$). Anal. ($\text{C}_{13}\text{H}_{11}\text{NO}$) C, H, N.

2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethylamine (21). Compound 21 was prepared in 81% yield from 20 by a method similar to that described for 11 as an oil. ^1H NMR (CDCl_3): δ 1.42–2.35 (m, 4H), 2.64–2.98 (m, 4H), 3.01–3.38 (m, 3H), 4.41–4.70 (m, 2H), 6.61 (d, 1H, $J = 8.1$), 6.95 (d, 1H, $J = 8.1$), hidden (2H).

N-[2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]acetamide (22a). Acetic anhydride (0.050 mL, 0.528 mmol) was added all at once to a vigorously stirred solution of 21 (71.6 mg, 0.352 mmol) and aqueous 1 N NaOH (1.5 mL) in tetrahydrofuran (1.5 mL) at room temperature. After the mixture was stirred for 30 min at room temperature, ethyl acetate and water were added. The organic layer was washed with brine and dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and hexane to yield 57.0 mg (66%) of 22a; mp 78–79 °C (from ethyl acetate/hexane). ^1H NMR (CDCl_3): δ 1.53–2.12 (m, 3H), 1.96 (s, 3H), 2.20–2.38 (m, 1H), 2.70–2.96 (m, 2H), 3.02–3.40 (m, 5H), 4.45–4.68 (m, 2H), 5.46 (br s, 1H), 6.62 (d, 1H, $J = 8.0$), 6.96 (d, 1H, $J = 8.0$). Anal. ($\text{C}_{18}\text{H}_{20}\text{NO}_2$) C, H, N.

N-[2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]propionamide (22b). Compound 22b was prepared in 78% yield from propionyl chloride and 21 by a method similar to that described for 22a; mp 102–104 °C (from isopropyl ether/hexane). ^1H NMR (CDCl_3): δ 1.14 (t, 3H, $J = 7.6$), 1.55–2.38 (m, 4H), 2.18 (q, 2H, $J = 7.6$), 2.69–2.99 (m, 2H), 3.02–3.40 (m, 5H), 4.42–4.63 (m, 2H), 5.61 (br s, 1H), 6.62 (d, 1H, $J = 7.8$), 6.95 (d, 1H, $J = 7.8$). Anal. ($\text{C}_{19}\text{H}_{21}\text{NO}_2$) C, H, N.

N-[2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]butanamide (22c). Compound 22c was prepared in 87% yield from butyryl chloride and 21 by a method similar to that described for 22a; mp 55–57 °C (from ethyl acetate). ^1H NMR (CDCl_3): δ 0.94 (t, 3H, $J = 7.3$), 1.51–1.90 (m, 4H), 1.92–2.08 (m, 1H), 2.12 (t, 2H, $J = 7.3$), 2.17–2.38 (m, 1H), 2.68–2.98 (m, 2H), 3.00–3.40 (m, 5H), 4.41–4.68 (m, 2H), 5.43 (br s, 1H), 6.62 (d, 1H, $J = 8.0$), 6.96 (d, 1H, $J = 8.0$). Anal. ($\text{C}_{19}\text{H}_{23}\text{NO}_2$) C, H, N.

N-[2-(5-Bromo-6-methoxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (27). Compound 27 was prepared in 86% yield from bromine and N-[2-(6-methoxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (26) by a method similar to that described for 15; mp 105–107 °C (from ethyl acetate). ^1H NMR (CDCl_3): δ 1.16 (t, 3H, $J = 7.6$), 1.49–1.82 (m, 2H), 1.98–2.41 (m, 2H), 2.21 (q, 2H, $J = 7.6$), 2.71–2.90 (m, 2H), 3.00–3.20 (m, 1H), 3.39 (q, 2H, $J = 7.1$), 3.88 (s, 3H), 5.50 (br s, 1H), 6.78 (s, 1H), 7.37 (s, 1H). Anal. ($\text{C}_{18}\text{H}_{20}\text{BrNO}_2$) C, H, N.

N-[2-(5-Bromo-6-hydroxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (28). A solution of 27 (26.0 g, 79.7 mmol) in dichloromethane (400 mL) was cooled to –20 °C in a solid CO_2 – CCl_4 bath, and BBr_3 (40.1 g, 0.160 mol) was then added slowly by syringe. The reaction was allowed to proceed for 1 h as the cooling bath gradually warmed to room temperature. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate as eluent to yield 21.4 g (86%) of 28; mp 149–151 °C (from ethyl

acetate). ^1H NMR (CDCl_3): δ 1.16 (t, 3H, $J = 7.5$), 1.50–1.80 (m, 2H), 1.90–2.12 (m, 1H), 2.20–2.40 (m, 1H), 2.24 (q, 2H, $J = 7.5$), 2.65–2.95 (m, 2H), 3.00–3.18 (m, 1H), 3.38 (q, 2H, $J = 7.1$), 5.82 (br s, 1H), 6.86 (s, 1H), 7.27 (s, 1H), hidden (1H). Anal. ($\text{C}_{18}\text{H}_{18}\text{BrNO}_2$) C, H, N, Br.

N-[2-(5-Bromo-6-(isopropenyloxy)-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (29). A 60% suspension of sodium hydride in mineral oil (0.648 g, 16.2 mmol) was added slowly to an ice-cooled solution of 28 (4.21 g, 13.5 mmol) in DMF (50 mL). After 30 min, by which time gas evolution had ceased, 3-chloro-2-methylpropane (3.07 g, 40.5 mmol) was added cautiously, and stirring was continued at 0 °C for 90 min. The mixture was made slightly acidic by the careful addition of a few drops of dilute HCl and then partitioned between ethyl acetate and H_2O . The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (1:2) as eluent to yield 4.15 g (84%) of 29; mp 105–108 °C (from ethyl acetate/hexane). ^1H NMR (CDCl_3): δ 1.16 (t, 3H, $J = 7.6$), 1.86 (s, 3H), 1.9–2.4 (m, 6H), 2.80 (m, 2H), 3.08 (m, 1H), 3.38 (q, 2H, $J = 7.6$), 4.47 (s, 2H), 5.00 (s, 1H), 5.17 (s, 1H), 5.40 (br s, 1H), 6.76 (s, 1H), 7.37 (s, 1H). Anal. ($\text{C}_{18}\text{H}_{24}\text{BrNO}_2$) C, H, N, Br.

N-[2-(5-Bromo-6-hydroxy-7-(2-methyl-2-propenyl)-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (30). A suspension of 29 (4.29 g, 11.7 mmol) in *N,N*-diethylaniline (30 mL) was heated at 200–205 °C for 2.5 h under an atmosphere of argon. The excess *N,N*-diethylaniline was removed in vacuo. The residual oil was taken up in ethyl acetate, washed successively with a brine solution and H_2O , and then dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (2:1) as eluent to yield 3.90 g (91%) of 30; mp 89–91 °C (from ethyl acetate/hexane). ^1H NMR (CDCl_3): δ 1.14 (t, 3H, $J = 7.6$), 1.40–1.80 (m, 2H), 1.80 (s, 3H), 1.90–2.10 (m, 2H), 2.17 (q, 2H, $J = 7.6$), 2.60–3.50 (m, 7H), 4.49 (s, 1H), 4.70 (s, 1H), 5.32 (br s, 1H), 5.47 (s, 1H), 7.21 (s, 1H). Anal. ($\text{C}_{18}\text{H}_{24}\text{BrNO}_2$) C, H, N, Br.

N-[2-(2,2-Dimethyl-1,6,7,8-tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]propionamide (31). Boron trifluoride diethyl etherate (4.12 mL, 32.5 mmol) was added slowly to an ice-cooled solution of 30 (2.38 g, 6.50 mmol) in dichloromethane (40 mL), and stirring was continued at 0 °C for 3 h. The reaction mixture was poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water and saturated aqueous NaHCO_3 and dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and isopropyl ether to yield 2.12 g (89%) of N-[2-(4-bromo-2,2-dimethyl-1,6,7,8-tetrahydro-2H-indeno[5,4-b]furan-8-yl)ethyl]propionamide; mp 98–101 °C (from ethyl acetate/isopropyl ether). ^1H NMR (CDCl_3): δ 1.15 (t, 3H, $J = 7.5$), 1.48 (s, 3H), 1.54 (s, 3H), 1.76–2.02 (m, 2H), 2.19 (q, 2H, $J = 7.5$), 2.25–2.38 (m, 1H), 2.62–3.16 (m, 6H), 3.32 (q, 2H, $J = 5.3$), 5.41 (br s, 1H), 7.11 (s, 1H). Anal. Calcd for $\text{C}_{18}\text{H}_{24}\text{BrNO}_2$: C, 59.02; H, 6.60; N, 3.82; Br, 21.81. Found: C, 58.94; H, 6.48; N, 3.98; Br, 21.97.

A solution of the propionamide (1.06 g, 2.90 mmol) and Et_3N (0.808 mL, 5.80 mmol) in ethanol (15 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (0.11 g, containing 50% water) in a hydrogen atmosphere at room temperature for 1 h and 50 °C for 30 min. After hydrogen absorption had ceased, the catalyst was removed by filtration. The filtrate was concentrated and recrystallized from a mixture of ethyl acetate and isopropyl ether to yield 0.635 g (76%) of 31; mp 69–72 °C (from ethyl acetate/isopropyl ether). ^1H NMR (CDCl_3): δ 1.14 (s, 3H), 1.43 (s, 3H), 1.50 (s, 3H), 1.60–2.10 (m, 2H), 2.13 (q, 2H, $J = 7.5$), 2.24–2.40 (m, 1H), 2.60–3.20 (s, 6H), 3.35 (q, 2H, $J = 5.3$), 5.39 (br s, 1H), 6.55 (d, 1H, $J = 7.6$), 6.95 (d, 1H, $J = 7.6$). Anal. ($\text{C}_{18}\text{H}_{26}\text{NO}_2$) C, H, N.

2-(6,7-Dimethoxy-2,3-dihydro-1H-inden-1-ylidene)acetonitrile (33). Compound 33 was prepared in 81% yield

from 6,7-dimethoxy-1-indanone³⁴ (32) and diethyl cyanomethylphosphonate by a method similar to that described for 10: mp 111–113 °C (from ethyl acetate). ¹H NMR (CDCl₃): δ 2.95–3.15 (m, 4H), 3.87 (s, 3H), 3.91 (s, 3H), 6.24 (t, 1H, *J* = 2.4), 6.95 (d, 1H, *J* = 8.6), 7.00 (d, 1H, *J* = 8.6). Anal. (C₁₅H₁₃NO₂) C, H, N.

2-(6,7-Dimethoxy-2,3-dihydro-1*H*-inden-1-yl)ethylamine (34). A solution of 33 (20.0 g, 92.9 mmol) in ethanol (760 mL) saturated with NH₃ gas was subjected to hydrogenation in the presence of Raney-cobalt (45 g washed with ethanol two times before use, ODHT-60, available through Kawaken Co.) in a hydrogen atmosphere (480 kPa) at room temperature for 7 h. After hydrogen absorption had ceased, the catalyst was removed by filtration. The filtrate was concentrated to yield 20.4 g (quant) of (*E*)-2-(6,7-dimethoxyindan-1-ylidene)-ethylamine as an oil. (*E*)-2-(6,7-Dimethoxyindan-1-ylidene)-ethylamine (10.0 g, 45.0 mmol) was dissolved in ethanol (100 mL), and the mixture was subjected to hydrogenation in the presence of 10% palladium on carbon (1.0 g, containing 50% water) in a hydrogen atmosphere at room temperature for 2 h. After absorption of the theoretical volume of hydrogen, the catalyst was removed by filtration. The filtrate was concentrated to yield 20.6 g (quant) of 34 as an oil, which was used directly in the next step. This oil was dissolved in ethanol saturated with HCl gas. The resulting solid was collected and recrystallized from ethanol to yield 34·HCl (78%); mp 141–143 °C (from ethanol). ¹H NMR (DMSO-*d*₆): δ 1.59–1.83 (m, 2H), 1.95–2.26 (m, 2H), 2.60–2.94 (m, 4H), 3.21–3.41 (m, 1H), 3.75 (s, 3H), 3.76 (s, 3H), 6.83 (d, 1H, *J* = 8.4), 6.89 (d, 1H, *J* = 8.4), 7.99 (br s, 2H). Anal. (C₁₅H₁₉NO₂·HCl) C, H, N, Cl.

***N*-(2-(6,7-Dimethoxy-2,3-dihydro-1*H*-inden-1-yl)ethyl)propionamide (35).** A solution of propionyl chloride (3.89 g, 42.0 mmol) was added dropwise to an ice-cooled solution of 34 (7.15 g, 32.3 mmol) and triethylamine (6.54 g, 64.6 mmol) in tetrahydrofuran (80 mL). After the mixture was stirred for 3 h at room temperature, ethyl acetate and water were added. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with methanol/ethyl acetate (1:99) as eluent to yield 7.72 g (86%) of 35; mp 90–92 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.14 (t, 3H, *J* = 7.7), 1.70–1.94 (m, 3H), 2.10–2.36 (m, 1H), 2.18 (q, 2H, *J* = 7.7), 2.65–3.20 (m, 3H), 3.25–3.55 (m, 2H), 3.85 (s, 3H), 3.87 (s, 3H), 5.90 (br s, 1H), 6.75 (d, 1H, *J* = 8.0), 6.90 (d, 1H, *J* = 8.0). Anal. (C₁₆H₂₃NO₃) C, H, N.

***N*-(2-(6,7-Dihydroxy-2,3-dihydro-1*H*-inden-1-yl)ethyl)propionamide (36).** Compound 36 was prepared in 73% yield from 35 and BBr₃ by a method similar to that described for 28; mp 98–101 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.21 (t, 3H, *J* = 7.5), 1.60–1.98 (m, 3H), 2.10–2.30 (m, 1H), 2.31 (q, 2H, *J* = 7.5), 2.60–3.15 (m, 3H), 3.22–3.40 (m, 1H), 3.52–3.75 (m, 1H), 5.95 (s, 1H), 6.01 (br s, 1H), 6.63 (d, 1H, *J* = 7.9), 6.74 (d, 1H, *J* = 7.9), 9.62 (s, 1H). Anal. (C₁₄H₁₉NO₃) C, H, N.

***N*-(2-(7,8-Dihydro-6*H*-indeno[4,5-*d*][1,3]dioxol-8-yl)ethyl)propionamide (37).** A 60% suspension of sodium hydride in mineral oil (0.300 g, 7.50 mmol) was added to an ice-cooled solution of hexamethyl phosphoramide (5 mL). This mixture was stirred for 10 min, and then, a solution of 36 (0.850 g, 3.41 mmol) in hexamethyl phosphoramide (5 mL) was added dropwise at room temperature over a period of 6 min. After gas evolution had subsided, diiodomethane (1.10 g, 4.10 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. Then, the mixture was made slightly acidic by the careful addition of a few drops of dilute HCl and partitioned between ethyl acetate and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate as eluent to yield 280 mg (31%) of 37; mp 102–104 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, *J* = 7.7), 1.70–1.89 (m, 2H), 1.90–2.10 (m, 1H), 2.15–2.40 (m, 1H), 2.20 (q, 2H, *J* = 7.7), 2.68–3.00 (m, 2H), 3.13–3.36 (m, 2H), 3.40–

3.59 (m, 1H), 3.68 (br s, 1H), 5.92 (dd, 2H, *J* = 1.5, 9.9), 6.67 (s, 2H). Anal. (C₁₅H₁₉NO₃) C, H, N.

6-Methoxy-7-nitro-1-indanone (38). A solution of potassium nitrate (24.3 g, 0.240 mol) in concentrated sulfuric acid (100 mL) was added slowly to a solution of 23 (30.0 g, 0.185 mol) in concentrated sulfuric acid (130 mL) at –10 °C. The reaction mixture was stirred at –10 °C for 20 min. It was then poured into ice-water and extracted with ethyl acetate. The organic layer was washed with water and saturated aqueous NaHCO₃ and dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and hexane to yield 22.3 g (58%) of 38; mp 155–158 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 2.78 (t, 2H, *J* = 5.6), 3.13 (t, 2H, *J* = 5.6), 3.94 (s, 3H), 7.34 (d, 1H, *J* = 8.4), 7.56 (d, 1H, *J* = 8.4). Anal. (C₁₀H₉NO₃) C, H, N.

2-(6-Methoxy-7-nitro-2,3-dihydro-1*H*-inden-1-ylidene)-acetonitrile (39). Compound 39 was prepared in 84% yield from 38 and diethyl cyanomethylphosphonate by a method similar to that described for 10; mp 138–141 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 3.00–3.20 (m, 4H), 3.92 (s, 3H), 5.42 (t, 1H, *J* = 2.6), 7.14 (d, 1H, *J* = 8.6), 7.43 (d, 1H, *J* = 8.6). Anal. (C₁₂H₁₀N₂O₃) C, H, N.

2-(7-Amino-6-methoxy-2,3-dihydro-1*H*-inden-1-ylidene)-acetonitrile (40). Compound 40 was prepared in 79% yield from 39 by a method similar to that described for 14; mp 119–121 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 2.90–3.20 (m, 4H), 3.87 (s, 3H), 4.23 (br s, 2H), 5.60 (t, 1H, *J* = 2.2), 6.69 (d, 1H, *J* = 8.0), 6.84 (d, 1H, *J* = 8.0).

***N*-(2-(7-Amino-6-methoxy-2,3-dihydro-1*H*-inden-1-yl)ethyl)propionamide (41).** 3-(2-Aminoethyl)-5-methoxy-2,3-dihydro-1*H*-inden-4-ylamine was prepared from 40 and Raney-nickel by a method similar to that described for 11, which was used directly in the next step. Propionic acid (0.852 g, 11.5 mmol) was added slowly to an ice-cooled suspension of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (3.30 g, 17.2 mmol) and 1-hydroxybenzotriazole monohydrate (2.21 g, 14.4 mmol). The mixture was stirred at room temperature for 1 h and then recooled to 0 °C. A solution of 3-(2-aminoethyl)-5-methoxy-2,3-dihydro-1*H*-inden-4-ylamine (2.00 g, 9.70 mmol) in DMF (10 mL) was added, and the reaction temperature was raised to room temperature. After 30 min, the reaction mixture was poured into water and extracted with ethyl acetate. The extract was taken up in dilute HCl, and the water layer was basified with a 4 N aqueous solution of sodium hydroxide to pH 10. The mixture was then extracted several times with ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethanol/ethyl acetate (1:10) as eluent to yield 1.02 g (40%) of 41; mp 71–73 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 1.09 (t, 3H, *J* = 7.5), 1.6–2.0 (m, 3H), 2.12 (q, 2H, *J* = 7.5), 2.25 (m, 1H), 2.7–3.2 (m, 3H), 3.34 (q, 2H, *J* = 5.0), 3.80 (br s, 2H), 3.83 (s, 3H), 5.67 (br s, 1H), 6.59 (d, 1H, *J* = 8.0), 6.66 (d, 1H, *J* = 8.0).

***N*-(2-(7-Amino-6-hydroxy-2,3-dihydro-1*H*-inden-1-yl)ethyl)propionamide (42).** Compound 42 was prepared in 88% yield from 41 and BBr₃ by a method similar to that described for 28 as an oil. ¹H NMR (CDCl₃): δ 1.11 (t, 3H, *J* = 7.5), 1.60–2.00 (m, 3H), 2.14 (q, 2H, *J* = 7.5), 2.23 (m, 1H), 2.70–2.90 (m, 2H), 3.19 (m, 1H), 3.34 (q, 2H, *J* = 5.1), 4.10 (br s, 2H), 5.69 (br s, 1H), 6.52 (d, 1H, *J* = 7.6), 6.60 (d, 1H, *J* = 7.6), hidden (1H).

***N*-(2-(7,8-Dihydro-6*H*-indeno[4,5-*d*][1,3]oxazol-8-yl)ethyl)propionamide (43).** A solution of methyl orthoformate (7.36 mL, 67.3 mmol) and methanol (1.4 mL) saturated with HCl gas was added dropwise to an ice-cooled solution of 42 (670 mg, 2.70 mmol) in methanol (5 mL). The mixture was stirred at room temperature for 30 min and then at 60 °C for 1 h. The reaction mixture was poured into ice-water and extracted with chloroform. The organic layer was washed with water and saturated aqueous NaHCO₃ and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with

chloroform/methanol (20:1) as eluent to yield 425 mg (61%) of 43; mp 81–84 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 1.20 (t, 3H, J = 7.5), 1.80–2.10 (m, 3H), 2.27 (q, 2H, J = 7.5), 2.37–2.53 (m, 1H), 2.80–3.20 (m, 3H), 3.55–3.80 (m, 2H), 6.93 (br s, 1H), 7.25 (d, 1H, J = 8.8), 7.40 (d, 1H, J = 8.8), 8.09 (s, 1H). Anal. (C₁₅H₁₆N₂O₂) C, H, N.

N-[2-(5-Bromo-6-(2-propynyloxy)-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (44). Compound 44 was prepared in 99% yield from 28 and propargyl bromide by a method similar to that described for 29; mp 104–107 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, J = 7.6), 1.50–2.40 (m, 6H), 2.55 (t, 1H, J = 2.3), 2.7–3.2 (m, 3H), 3.38 (t, 2H, J = 7.5), 4.76 (d, 2H, J = 2.3), 5.48 (br s, 1H), 6.93 (s, 1H), 7.38 (s, 1H). Anal. (C₁₇H₂₀BrNO₂) C, H, N, Br.

N-[2-(5-Bromo-3,7,8,9-tetrahydrocyclopenta[f]chromen-9-yl)ethyl]propionamide (45). A mixture of 44 (2.94 g, 8.40 mmol) in bromobenzene (30 mL) was heated at 200 °C for 18 h in a stainless steel bomb. The reaction mixture was cooled to room temperature, and the solvent was evaporated to dryness in vacuo. The residue was purified by column chromatography with ethyl acetate as eluent to yield 2.59 g (88%) of 45; mp 110–111 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.14 (t, 3H, J = 7.5), 1.50–2.50 (m, 5H), 2.60–3.10 (m, 3H), 3.15–3.25 (m, 1H), 3.32 (q, 2H, J = 7.5), 4.80–4.90 (m, 2H), 5.40 (br s, 1H), 5.88 (dt, 1H, J = 10, 3.8), 6.45 (dd, 1H, J = 1.6, 9.8), 7.18 (s, 1H). Anal. (C₁₇H₂₀BrNO₂) C, H, N, Br.

N-[2-(1,2,3,7,8,9-Hexahydrocyclopenta[f]chromen-9-yl)ethyl]propionamide (46). A solution of 45 (210 mg, 0.600 mmol) in ethanol (5 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (200 mg, containing 50% water) in a hydrogen atmosphere at room temperature for 3 h. After hydrogen absorption had ceased, the catalyst was removed by filtration. The filtrate was concentrated, and the residue was purified by column chromatography with hexane/ethyl acetate (1:1) as eluent to yield 139 mg (65%) of 46; mp 85–88 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, J = 7.6), 1.80–2.10 (m, 6H), 2.15 (q, 2H, J = 7.6), 2.60–3.50 (m, 7H), 4.00–4.30 (m, 2H), 5.35 (br s, 1H), 6.63 (d, 1H, J = 8.2), 6.94 (d, 1H, J = 8.2). Anal. (C₁₇H₂₂NO₂) C, H, N.

8-Hydroxy-7-nitro-1-indanone (48). Compound 48 was prepared in 61% yield from 47 and potassium nitrate by a method similar to that described for 38; mp 218–220 °C (from ethanol/hexane). ¹H NMR (CD₃OD): δ 2.37 (t, 2H, J = 5.5), 2.74 (t, 2H, J = 5.5), 2.95 (s, 1H), 6.95 (d, 1H, J = 8.4), 7.15 (d, 1H, J = 8.4).

Ethyl 2-[(4-Nitro-3-oxo-2,3-dihydro-1H-inden-5-yl)oxy]acetate (49). Compound 49 was prepared in 94% yield from 48 and ethyl bromoacetate by a method similar to that described for 29; mp 137–139 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.29 (t, 3H, J = 7.1), 2.79 (t, 2H, J = 6.0), 3.14 (t, 2H, J = 6.0), 4.25 (q, 2H, J = 7.1), 4.74 (s, 2H), 7.25 (d, 1H, J = 8.4), 7.55 (d, 1H, J = 8.4).

Ethyl 2-[(4-Amino-3-oxo-2,3-dihydro-1H-inden-5-yl)oxy]acetate (50). Compound 50 was prepared in 98% yield from 49 by a method similar to that described for 14 and used in the next reaction without further purification. ¹H NMR (CDCl₃): δ 1.29 (t, 3H, J = 7.1), 2.30–3.00 (m, 4H), 4.28 (q, 2H, J = 7.1), 4.61 (s, 2H), 5.89 (br s, 2H), 6.53 (d, 1H, J = 8.2), 6.87 (d, 1H, J = 8.2).

7,8-Dihydroindeno[5,4-b][1,4]oxazine-2,9(1H,3H)-dione (51). A mixture of 50 (8.70 g, 34.9 mmol) and potassium *tert*-butoxide (404 mg, 3.60 mmol) in toluene (200 mL) was heated at reflux for 12 h under an atmosphere of argon. The reaction mixture was cooled to room temperature and poured into water. It was then made slightly acidic by careful addition of a few drops of dilute HCl and partitioned between ethyl acetate and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate/hexane (1:1) as eluent to yield 4.8 g (66%) of 51; mp 136–139 °C (from ethyl acetate/hexane).

¹H NMR (CDCl₃): δ 2.74 (t, 2H, J = 5.8), 3.10 (t, 2H, J = 5.8), 4.68 (s, 2H), 7.01 (d, 1H, J = 7.2), 7.17 (d, 1H, J = 7.2), 9.52 (br s, 1H).

2-(2-Oxo-2,3,7,8-tetrahydroindeno[5,4-b][1,4]oxazin-9(1H)-ylidene)acetonitrile (52). Compound 52 was prepared in 86% yield from 51 and diethyl cyanomethylphosphonate by a method similar to that described for 10; mp 158–161 °C (from chloroform). ¹H NMR (CDCl₃): δ 3.00–3.20 (m, 4H), 4.62 (s, 2H), 5.62 (t, 1H, J = 2.3), 6.97 (d, 1H, J = 8.2), 7.06 (d, 1H, J = 8.2), 8.07 (br s, 1H). Anal. (C₁₃H₁₀N₂O₂) C, H, N.

9-(2-Aminocetyl)-1,7,8,9-tetrahydroindeno[5,4-b][1,4]oxazin-2(3H)-one (53). A solution of 52 (3.00 g, 13.3 mmol) in ethanol (300 mL) saturated with NH₃ gas was subjected to hydrogenation in the presence of Raney-nickel (14 g washed with ethanol two times before use) in a hydrogen atmosphere (480 kPa) at 40 °C for 6 h. After hydrogen absorption had ceased, the catalyst was removed by filtration and the filtrate was concentrated. The residue was taken up into 50 mL of ethyl acetate, and the product was extracted with 50 mL of 2 N HCl. The aqueous extracts were combined and rendered strongly basic with the addition of a 4 N aqueous solution of sodium hydroxide to pH 10. The free base was then extracted several times with chloroform/methanol (10:1). The combined extracts were dried over anhydrous magnesium sulfate. The filtrate was concentrated to give a solid, which was recrystallized from a mixture of ethyl acetate and isopropyl ether to yield 1.95 g (62%) of 53; mp 128–134 °C (from ethyl acetate/isopropyl ether). ¹H NMR (CDCl₃): δ 1.40–1.90 (m, 6H), 2.20–2.50 (m, 2H), 2.70 (dd, 1H, J = 8.0, 15.4), 2.90–3.00 (m, 2H), 3.40 (q, 1H, J = 7.9), 4.44 (d, 1H, J = 15.0), 4.58 (d, 1H, J = 15.0), 6.75 (d, 1H, J = 8.0), 6.79 (d, 1H, J = 8.0). Anal. (C₁₃H₁₆N₂O₂·0.25H₂O) C, H, N.

2-(1,2,3,7,8,9-Hexahydroindeno[5,4-b][1,4]oxazin-9-yl)-ethylamine (54). A solution of 53 (1.23 g, 5.30 mmol) was dissolved in dry tetrahydrofuran (30 mL) and added dropwise to a stirring ice-cooled suspension of LiAlH₄ (0.812 g, 21.4 mmol) in tetrahydrofuran (5 mL). After the addition was complete, the mixture was heated at reflux for 18 h under an atmosphere of argon. The mixture was then cooled in an ice bath, and the excess LiAlH₄ was decomposed by the careful addition of water (4 mL). The mixture was filtered through a Celite pad, the filter cake was rinsed well with ethyl acetate, and the solvent was removed by rotary vacuum evaporation. The residue was taken up into 50 mL of ethyl acetate, and the product was extracted with 50 mL of 2 N HCl. The aqueous extracts were combined and rendered strongly basic with the addition of a 4 N aqueous solution of sodium hydroxide to pH 10. The free base was then extracted several times with chloroform/methanol (10:1). The combined extracts were dried over anhydrous magnesium sulfate. The filtrate was concentrated to yield 0.93 g (80%) of 54 as an oil, which was used directly in the next step. ¹H NMR (CDCl₃): δ 1.10–3.20 (m, 12H), 3.41 (m, 2H), 4.20 (m, 2H), 6.49 (d, 1H, J = 8.0), 6.61 (d, 1H, J = 8.0).

N-[2-(1,2,3,7,8,9-Hexahydroindeno[5,4-b][1,4]oxazin-9-yl)ethyl]propionamide (55). Propionic acid (0.134 mL, 1.80 mmol) was added slowly to an ice-cooled suspension of WSC (0.518 g, 2.70 mmol) and HOBt (0.352 g, 2.30 mmol). The mixture was stirred at room temperature for 1 h and then recooled to 0 °C. A solution of 54 (0.393 g, 1.80 mmol) in DMF (15 mL) was added, and the reaction temperature was raised to room temperature. After 30 min, the reaction mixture was poured into water and extracted with ethyl acetate. The combined extracts were dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate/ethanol (10:1) as eluent to yield 252 mg (51%) of 55; mp 80–83 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.11 (t, 3H, J = 7.5), 1.50–2.30 (m, 6H), 2.60–3.20 (m, 3H), 3.32 (q, 2H, J = 6.7), 3.43 (t, 2H, J = 4.4), 3.85 (br s, 1H), 4.20 (t, 2H, J = 4.4), 5.84 (br s, 1H), 6.50 (d, 1H, J = 8.0), 6.62 (d, 1H, J = 8.0). Anal. (C₁₆H₂₂N₂O₂) C, H, N.

N-[2-(2-Oxo-1,2,3,7,8,9-hexahydroindeno[5,4-b][1,4]oxazin-9-yl)ethyl]propionamide (56). Compound 56 was

prepared in 88% yield from 53 and propionic acid by a method similar to that described for 55; mp 216–219 °C (from ethyl acetate/methanol). ¹H NMR (CDCl₃): δ 1.18 (d, 3H, *J* = 7.5), 1.50–2.00 (m, 3H), 2.10–2.30 (m, 3H), 2.70–3.10 (m, 2H), 3.30–3.50 (m, 3H), 4.59 (s, 2H), 5.97 (br s, 1H), 6.81 (s, 2H), 9.77 (br s, 1H). Anal. (C₁₈H₂₀N₂O₃) C, H, N.

N-[2-(2,3,8,9-Tetrahydro-7H-indeno[4,5-*b*][1,4]dioxin-9-yl)ethyl]propionamide (57). A mixture of 36 (1.00 g, 4.01 mmol), dibromoethane (2.90 g, 15.4 mmol), potassium carbonate (1.63 g, 11.8 mmol), and copper(II) oxide (31.8 mg, 0.400 mmol) in DMF (15 mL) was heated at 140 °C for 6 h. The mixture was cooled to room temperature and poured into water. Then it was made slightly acidic by the careful addition of a few drops of dilute HCl and partitioned between ethyl acetate and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate as eluent to yield 718 mg (65%) of 57; mp 120–122 °C (from ethyl acetate/hexane). ¹H NMR (CDCl₃): δ 1.15 (t, 3H, *J* = 7.5), 1.60–2.00 (m, 3H), 2.10–2.32 (m, 1H), 2.19 (q, 2H, *J* = 7.5), 2.61–3.01 (m, 2H), 3.08–3.53 (m, 3H), 4.25 (br s, 4H), 5.67 (br s, 1H), 6.69 (s, 2H). Anal. (C₁₈H₂₁NO₃) C, H, N.

(S)-N-[2-(5-Bromo-6-methoxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (61). Compound 61 was prepared in 86% yield from 60 and bromine by a method similar to that described for 15; mp 105–107 °C (from ethyl acetate); [α]_D²⁰ +5.2° (c 1.000, ethanol). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, *J* = 7.7), 1.49–1.81 (m, 2H), 1.98–2.41 (m, 2H), 2.21 (q, 2H, *J* = 7.7), 2.69–2.98 (m, 2H), 3.00–3.20 (m, 1H), 3.39 (q, 2H, *J* = 7.3), 3.88 (s, 3H), 5.48 (br s, 1H), 6.78 (s, 1H), 7.37 (s, 1H). Anal. (C₁₈H₂₁BrNO₂) C, H, N.

(S)-N-[2-(5-Bromo-6-hydroxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (62). A solution of 61 (56.7 g, 174 mmol) in dichloromethane (400 mL) was cooled to –30 °C in a solid CO₂–acetone bath. BBr₃ (95.8 g, 382 mmol) was then added slowly by syringe. The reaction was stirred at –20 to –15 °C for 30 min. The reaction mixture was poured into ice-water and stirred for a further 10 min at room temperature. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate as eluent to yield 51.1 g (94%) of 62; mp 146–148 °C (from ethyl acetate); [α]_D²⁰ +2.7° (c 1.001, ethanol). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, *J* = 7.5), 1.50–1.80 (m, 2H), 1.90–2.12 (m, 1H), 2.20–2.40 (m, 1H), 2.24 (q, 2H, *J* = 7.5), 2.65–2.95 (m, 2H), 3.00–3.18 (m, 1H), 3.38 (q, 2H, *J* = 7.1), 5.82 (br s, 1H), 6.86 (s, 1H), 7.27 (s, 1H), hidden (1H). Anal. (C₁₇H₁₉BrNO₂) C, H, N.

(S)-N-[2-(6-Allyloxy-5-bromo-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (63). Compound 63 was prepared in 96% yield from 62 and allyl bromide by a method similar to that described for 29; mp 85–87 °C (from ethyl acetate/hexane); [α]_D²⁰ +3.7° (c 1.003, ethanol). ¹H NMR (CDCl₃): δ 1.16 (t, 3H, *J* = 7.5), 1.48–1.80 (m, 2H), 1.90–2.40 (m, 2H), 2.20 (q, 2H, *J* = 7.5), 2.70–2.91 (m, 2H), 3.00–3.20 (m, 1H), 3.37 (q, 2H, *J* = 7.4), 4.59 (m, 2H), 5.25–5.60 (m, 3H), 5.97–6.20 (m, 1H), 6.76 (s, 1H), 7.37 (s, 1H). Anal. (C₁₇H₂₂BrNO₂) C, H, N.

(S)-N-[2-(7-Allyl-5-bromo-6-hydroxy-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (64). Compound 64 was prepared in 80% yield from 63 and *N,N*-diethylaniline by a method similar to that described for 30; mp 85–87 °C (from ethyl acetate/hexane); [α]_D²⁰ –51.3° (c 1.003, ethanol). ¹H NMR (CDCl₃): δ 1.14 (t, 3H, *J* = 7.6), 1.45–2.13 (m, 4H), 2.18 (q, 2H, *J* = 7.6), 2.68–3.65 (m, 7H), 4.93–5.13 (m, 2H), 5.41 (br s, 1H), 5.49 (s, 1H), 5.89–6.10 (m, 1H), 7.20 (s, 1H). Anal. (C₁₇H₂₂BrNO₂) C, H, N, Br.

(S)-N-[2-(5-Bromo-6-hydroxy-7-(2-hydroxyethyl)-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (65). A stream of O₃ was bubbled through a solution of 64 (588 mg, 1.67 mmol) in methanol (30 mL), which was cooled to –78 °C until a blue color persisted. The excess ozone was removed by first purging with oxygen and then with nitrogen. Sodium borohydride (510

mg, 13.4 mmol) was added at about –70 °C to decompose ozonide, and the mixture was allowed to warm to room temperature with stirring for 1 h. Then, the mixture was made slightly acidic by the careful addition of a few drops of dilute HCl and partitioned between ethyl acetate/butanol (1:1) and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated to yield 0.589 g (99%) of 65, which was used directly in the next step. The analytical sample was obtained by recrystallization from a mixture of ethyl acetate and methanol; mp 85–87 °C (from ethyl acetate/methanol); [α]_D²⁰ –43.7° (c 1.002, ethanol). ¹H NMR (CDCl₃): δ 1.13 (t, 3H, *J* = 7.5), 1.40–2.10 (m, 4H), 2.17 (q, 2H, *J* = 7.5), 2.62–3.01 (m, 4H), 3.07–3.22 (m, 1H), 3.28 (q, 2H, *J* = 6.8), 3.89 (br s, 2H), 5.47 (t, 1H, *J* = 3.7), 6.31 (br s, 1H), 7.20 (s, 1H), 9.07 (s, 1H). Anal. (C₁₈H₂₂BrNO₃) C, H, N, Br.

(S)-N-[2-(6-Hydroxy-7-(2-hydroxyethyl)-2,3-dihydro-1H-inden-1-yl)ethyl]propionamide (66). A mixture of 65 (590 mg, 1.66 mmol) and triethylamine (184 mg, 1.82 mmol) in methanol (5 mL) was subjected to hydrogenation in the presence of 10% palladium on carbon (100 mg, containing 50% water) in a hydrogen atmosphere at room temperature. After absorption of the theoretical volume of hydrogen, the catalyst was removed by filtration. Then, the mixture was made slightly acidic by the addition of a few drops of dilute HCl and partitioned between ethyl acetate/butanol (1:1) and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated to yield 0.419 g (91%) of 66, which was used directly in the next step. The analytical sample was obtained by recrystallization from a mixture of ethyl acetate and methanol; mp 144–146 °C (from ethyl acetate/hexane); [α]_D²⁰ –69.7° (c 1.002, ethanol). ¹H NMR (CDCl₃): δ 1.12 (t, 3H, *J* = 7.7), 1.45–2.10 (m, 4H), 2.16 (q, 2H, *J* = 7.7), 2.60–3.00 (m, 4H), 3.10–3.23 (m, 1H), 3.29 (q, 2H, *J* = 6.8), 3.86 (q, 2H, *J* = 5.5), 5.00 (t, 1H, *J* = 4.4), 6.41 (br s, 1H), 6.69 (d, 1H, *J* = 7.9), 6.91 (d, 1H, *J* = 7.9), 8.86 (s, 1H). Anal. (C₁₈H₂₃NO₃) C, H, N.

(S)-N-[2-(1,6,7,8-Tetrahydro-2H-indeno[5,4-*b*]furan-8-yl)ethyl]propionamide ((S)-(-)-22b). Methanesulfonyl chloride (1.39 mL, 18.0 mmol) was added dropwise to a stirred solution of the alcohol 66 (4.99 g, 18.0 mmol) in pyridine (14.6 mL, 180 mmol) at about –10 °C. After the mixture was stirred for 25 min at –10 to –5 °C, an additional quantity of methanesulfonyl chloride (0.697 mL, 9.00 mmol) was added and the solution was stirred at –10 to –5 °C for another 25 min. A mixture of ethyl acetate (10 mL) and saturated aqueous NaHCO₃ (10 mL) was added slowly to the above solution at 0 °C. The solution was allowed to warm to room temperature and stirred for an additional 30 min. It was poured into water and made slightly acidic by the careful addition of dilute HCl and then partitioned between ethyl acetate and H₂O. The organic layer was washed with water and dried over anhydrous magnesium sulfate. The filtrate was concentrated, and the residue was purified by column chromatography with ethyl acetate as eluent to yield 4.02 g (86%) of (S)-(-)-22b; mp 113–115 °C (from ethyl acetate); [α]_D²⁰ –57.8° (c 1.004, chloroform). ¹H NMR (CDCl₃): δ 1.14 (t, 3H, *J* = 7.7), 1.52–2.40 (m, 4H), 2.17 (q, 2H, *J* = 7.7), 2.69–3.00 (m, 2H), 3.01–3.40 (m, 5H), 4.42–4.64 (m, 2H), 5.40 (br s, 1H), 6.62 (d, 1H, *J* = 7.7), 6.95 (d, 1H, *J* = 7.7). Anal. (C₁₈H₂₁NO₂) C, H, N.

Separation of Enantiomers by Chiral High-Performance Liquid Chromatography (HPLC). The racemic mixture 22b was optically resolved by high-performance column chromatography (apparatus, LC Module 1 (Nippon Millipore Ltd.); column, Ceramospher RU-1 (10 mm (id) × 250 mm, Shiseido); mobile phase, methanol; flow rate, 4.4 mL/min; column temperature, 50 °C; sample concentration, 17% (w/v); amount injected, 8.5 mg). Removal of the solvent gave the separate enantiomers, each of which was then examined by analytical chiral HPLC for purity. (R)-(+)-22b: [α]_D²⁰ +57.8° (c 1.005, chloroform). Anal. (C₁₈H₂₁NO₂) C, H, N.

Docking Study. The three-dimensional (3D) model of the human melatonin receptor (MT₁) was constructed by comparative modeling techniques from the crystal structure of bovine

rhodopsin (Protein Data Bank access number 1F88).³¹ Among the loop regions, only the second extracellular loop was included in the model, which is involved in ligand binding according to chimera receptor experiments.³² In the initial model, the second extracellular loop occludes the ligand binding pocket and should be drastically moved to make favorable interactions with ligands. We applied a simulated annealing procedure with van der Waals parameter scaling to change the conformation of the loop. Comparative modeling was carried out on a SiliconGraphics O²(C) workstation (Silicon Graphics Inc., Mountain View, CA) with the InsightII/Discover program package (Molecular Simulations Inc., San Diego, CA). The binding mode was analyzed by the program DOCK (version 4.01, UCSF, Kuntz, et al.) using standard parameters.

B. Pharmacology. Preparation of CHO Membranes for Receptor Binding Assays. cDNA encoding the human MT₁ gene was introduced into CHO cells. A cell line stably expressing the MT₁ receptor (CHO-hMelR7) was selected and cultured in Eagle's Minimum Essential Medium- α (MEM- α) supplemented with 10% dialyzed fetal bovine serum (dFBS) in a 5% CO₂/95% air atmosphere. Cells were harvested at confluence in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution containing 5 mM EDTA and collected by centrifugation. Cells were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25 °C), washed twice, pelleted, and stored at -30 °C until binding assays.

The day before transfection, CHO cells were grown in MEM- α supplemented with 10% dFBS until the cells were 80% confluent. The cells were transfected with pCMV-human MT₂ melatonin receptor expression vector by using Lipofectamine reagent (Invitrogen). Two days after the transfection, the cells were harvested in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution containing 5 mM EDTA and collected by centrifugation. Cells were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25 °C), washed twice, pelleted, and stored at -30 °C until binding assays.

Affinity for MT₁ and MT₂ Melatonin Receptor. The procedure of this binding assay was based on the method of Rivkees et al. with minor modifications.³⁵ The frozen homogenate was thawed, suspended in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25 °C), and used for the binding assay. For the assay using CHO-hMelR7 homogenate, 10 μ L of DMSO solution of a test compound was mixed with homogenate and 40 (MT₁) or 200 pM (MT₂) 2-[¹²⁵I]iodomelatonin in a total volume of 1 mL and incubated at 25 °C for 150 min. The reaction was terminated by addition of 3 mL of ice-cold buffer followed by vacuum filtration on Whatman GF/B. The filter was further washed twice with 3 mL of ice-cold buffer, and the radioactivity was measured by a γ -counter. Nonspecific binding was defined in the presence of 10 μ M melatonin. The 50% inhibitory concentration (IC₅₀) was calculated using log-probit analysis. The dissociation constant of the compound for the receptor (K_i) was calculated using the following equation:

$$K_i = IC_{50} / (1 + L/K_d)$$

where L and K_d represent the concentration and the affinity constant of 2-[¹²⁵I]melatonin in the binding assay, respectively.

Affinity for Low Affinity Melatonin Binding Sites (MT₂ Site and Others) in Syrian Hamster Brain and Peripheral Organs. The procedure of this binding assay was based on the method of Niles et al.³⁶ with minor modifications. The specific binding of 2-[¹²⁵I]iodomelatonin to the hamster MT₂ receptor was saturable. The brain, liver, kidney, and spleen were dissected from male Syrian hamsters (7–8 weeks old) and homogenized in 50 mM Tris-HCl (pH 7.4 at 4 °C). After the removal of connective tissues by filtration using a quadruple layer of cotton gauze, the homogenate was centrifuged at 48 000g for 10 min. The resultant pellet was washed twice with ice-cold buffer by resuspension and recentrifugation, and the final pellet was suspended in the same buffer. An aliquot of the homogenate was incubated with 200 pM 2-[¹²⁵I]iodomelatonin and a test compound at 4 °C for 60 min in a total volume of 200 μ L. The reaction was terminated by

addition of 3 mL of ice-cooled assay buffer followed by rapid vacuum filtration on Whatman GF/B. The filter was washed a further two times with 3 mL of ice-cooled buffer and placed in a polystyrene tube. The radioactivity was measured with a γ -counter. Nonspecific binding was determined in the presence of 10⁻⁴ M melatonin. The IC₅₀ value was calculated by log-probit analysis.

Inhibition of Forskolin-Induced cAMP Formation in CHO Cells Expressing Human Melatonin Receptor (MT₁ Site). CHO cells transfected with the human melatonin receptor (MT₁) gene were plated in MEM- α supplemented with 10% dFBS at a density of 5 $\times$ 10⁴ cells/well (1.3 $\times$ 10⁴ cells/cm²) on Iwaki 12 well plates and cultured for 3 days. Cells were washed twice with 1 mL of modified Hanks' balanced salt solution (with 10 mM HEPES-Na, pH 7.3, without NaHCO₃) and then 0.5 mL of the same solution containing 100 μ M 3-isobutyl-1-methyl xanthine (IBMX), and a test compound was added. After the cells were preincubated for 6 min at 37 °C, 10 μ L of forskolin (final concentration of 1 μ M) was added and the cells were incubated further for 15 min. The reaction was terminated by the addition of 50 μ L of 55% perchloric acid. An aliquot (200 μ L) of the reaction mixture was transferred to a test tube, neutralized with 100 μ L of 1.6 M NaOH solution, and assayed for cAMP concentration using an Amersham cAMP radioimmunoassay system. The IC₅₀ value was calculated by log-probit analysis.

Effects of (S)-(-)-22b on Sleep and Wakefulness in Cats. Eight adult cats (three males and five females) weighing 2.5–5.5 kg at the time of surgery were used. The cats were housed individually in a room maintained at 24 $\pm$ 1 °C with a 12 h light/dark cycle (light on 7:00 a.m.). They were fed once daily (9:00 a.m.), and water was available ad libitum. On the day of the experiment, however, they were fed after completion of the experiment. Cats were mounted on a stereotaxic apparatus under pentobarbital (40 mg/kg, ip) anesthesia. Electrodes were implanted bilaterally in the frontal and parietal cortices and hippocampus according to the cat brain atlas of Snider and Niemer.³⁷ As cortical electrodes, stainless steel screws were used. The depth bipolar recording electrode was made up of twisted stainless steel wires (0.3 mm in diameter) insulated except at the tips (0.5 mm). Ocular movement was recorded from stainless steel screws fixed over the bony orbit. The effect of (S)-(-)-22b on sleep was investigated by electroencephalogram (EEG) recording. The cats were allowed to recover from surgery for at least 7 days before habituation to the test chamber and EEG recording. The test chamber (650 cm $\times$ 35 cm $\times$ 45 cm) was constructed entirely of metal except for one Plexiglass wall and was located in a constantly illuminated, ventilated, sound-proof, and electrically shielded room. The cats were well-accustomed to the test chamber and EEG recording before the test. A cat was transferred to the test chamber in the experimental room, and the cables were attached. EEGs, electrooculograms (EOG), and electromyograms (EMG) were recorded for 9 h (1 h before to 8 h after treatment with vehicle, (S)-(-)-22b or melatonin). During the recording, cats were observed from outside the experimental room on a video monitor and their behavioral and postural changes were recorded continuously throughout the experiment. Each potential amplified and filtered with a polygraph (Nihondenki-Sanei) was recorded by a magnetic pen recorder. An EEG power spectral analysis was also performed continuously using a fast Fourier transform (FFT) system equipped with a personal computer (NEC, PC-9801) and recorded on a MO disk. The state of the cat was classified into three stages based on the following criteria: wakefulness (marked tonic EMG activity, low-voltage fast cortical EEG with a low power spectrum of δ -waves, a regular hippocampal θ -rhythm, and slow EOG activity); SWS (markedly reduced EMG activity, spindles and slow waves of high-voltage cortical EEG with a high power spectrum of δ -waves, and reduced EOG activity); REM (an almost complete absence of EMG activity, low-voltage fast cortical EEG with a low power spectrum of δ -waves, an extremely regular hippocampal θ -rhythm, and a frequently observed high-voltage EOG caused

by REM). (S)-(-)-22b and melatonin were suspended in 0.5% methyl cellulose (MC). Each cat was administered orally with a capsule containing the (S)-(-)-22b or melatonin solution. In the control trial, the cat was given a capsule containing vehicle. Each dose of (S)-(-)-22b or melatonin was compared with the counterbalanced control trial conducted before or after the drug trial (dependent sample). The duration between the trials was more than 7 days in order to exclude any effect of the former trial. For statistical analysis, a paired *t*-test was used.

Acknowledgment. We acknowledge the contributions of Mr. Koji Onishi and Mr. Norio Tada for the plasma metabolite analysis, Mr. Toshimasa Tanaka for the MO calculations, and Mr. Nobuhiro Fujii and Mr. Masaki Setoh for technical assistance.

Note Added after ASAP Posting. This manuscript was released ASAP on 8/15/2002 with an error in the compound designation for the eighth entry of Table 1. The correct version was posted on 9/5/2002.

References

- (1) (a) Goldman, B. D.; Darrow, J. M. The pineal gland and mammalian photoperiodism. *Neuroendocrinology* 1983, 37, 386-396. (b) Reiter, R. J. The melatonin message: Duration versus coincidence hypotheses. *Life Sci.* 1987, 40, 2119-2131. (c) Reiter, R. J. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. *Endocr. Rev.* 1991, 12, 151-180. (d) Hagan, K. M.; Oakley, N. R. Melatonin comes of age? *Trends Pharmacol. Sci.* 1995, 16, 81-83.
- (2) (a) Underwood, H.; Goldman, B. D. Vertebrate circadian and photoperiodic systems: role of the pineal gland and melatonin. *J. Biol. Rhythms* 1987, 2, 279-315. (b) Cassone, V. M. Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.* 1990, 13, 457-464. (c) Armstrong, S. M. Melatonin and circadian control in mammals. *Experientia* 1989, 45, 932-938.
- (3) Wechmann, A. F. Melatonin: parallels in pineal gland and retina. *Exp. Eye Res.* 1986, 42, 507-527.
- (4) (a) Moore, R. Y.; Klein, D. C. Visual pathways and the central neural control of a circadian rhythm in pineal serotonin *N*-acetyltransferase activity. *Brain Res.* 1974, 71, 17-33. (b) Reppert, S. M.; Perlow, M. J.; Ungerleider, L. G.; Mishkin, M.; Tamarkin, L.; Orioff, D. G.; Hoffman, H. J.; Klein, D. C. Effects of damage to the suprachiasmatic area of the anterior hypothalamus on the daily melatonin and cortisol rhythms in the rhesus monkey. *J. Neurosci.* 1981, 1, 1414-1425. (c) Moore, R. Y. Neural control of the pineal gland. *Behav. Brain Res.* 1996, 73, 125-130.
- (5) (a) Lynch, H. J.; Ozaki, Y.; Shakai, D.; Wurtman, R. J. Melatonin excretion of man and rats: effect of time of day, sleep, pinealectomy and food consumption. *Int. J. Biometeorol.* 1975, 19, 267-279. (b) Jlmerson, D. C.; Lynch, H. J.; Post, R. M.; Wurtman, R. J.; Bunney, W. E. Urinary melatonin rhythms during sleep deprivation in depressed patients and normals. *Life Sci.* 1977, 20, 1501-1508.
- (6) (a) Aakerstedt, T.; Freeberg, J. E.; Friberg, Y.; Wetterberg, L. Melatonin excretion, body temperature and subjective arousal during 64 h of sleep deprivation. *Psychoneuroendocrinology* 1978, 4, 219-225. (b) Nakagawa, H.; Sack, R. L.; Lewy, A. J. Sleep propensity free-runs with the temperature, melatonin and cortisol rhythms in a totally blind person. *Sleep* 1992, 15, 330-336. (c) Tzischinsky, O.; Shlitzner, A.; Lavie, P. The association between the nocturnal sleep gate and nocturnal onset of urinary 6-sulfatoxymelatonin. *J. Biol. Rhythms* 1993, 8, 199-209. (d) Zhdanova, I. V.; Wurtman, R. J. Efficacy of melatonin as a sleep-promoting agent. *J. Biol. Rhythms* 1997, 12, 644-650.
- (7) (a) Wehr, T. A. The Durations of Human Melatonin Secretion and Sleep Respond to Changes in Daylength (Photoperiod). *J. Clin. Endocrinol. Metab.* 1991, 73, 1276-1280. (b) Olan, A.; Ferini-Strambi, L.; Zucconi, M.; Stankov, B.; Fraschini, F.; Smirne, S. Melatonin and delayed sleep phase syndrome: ambulatory polygraphic evaluation. *NeuroReport* 1994, 5, 132-134.
- (8) (a) Petrie, K.; Conaglen, J. V.; Thompson, L.; Chamberlain, K. Effect of melatonin on jet lag after long haul flights. *Br. Med. J.* 1989, 298, 705-707. (b) Arendt, J. Melatonin in humans: jet-lag and after. *Adv. Pineal Res.* 1991, 5, 289-302. (c) Nickelsen, T.; Lang, A.; Bergau, L. The effect of 6-, 9-, and 11-hour time shifts on circadian rhythms: adaptation of sleep parameters and hormonal patterns following the intake of melatonin or placebo. *Adv. Pineal Res.* 1991, 5, 303-306.
- (9) Sack, R. L.; Blood, M. L.; Lewy, A. J. Melatonin rhythms in night shift workers. *Sleep* 1992, 15, 434-441.
- (10) Rosenthal, N. E.; Sack, D. A.; Jacobsen, F. M.; James, S. P.; Parry, B. L.; Arendt, J.; Tamarkin, L.; Wehr, T. A. Melatonin in seasonal affective disorder and phototherapy. *J. Neurol. Transm. Suppl.* 1988, 21, 257-267.
- (11) (a) Nair, N. P. V.; Hariharasubramanian, N.; Pillai, C.; Isaac, L.; Thavundayil, J. X. Plasma melatonin - an index of brain aging in humans? *Biol. Psychiatry* 1988, 21, 141-150. (b) Waldhauser, F.; Weissenbacher, G.; Tatzler, E.; Clsinger, B.; Waldhauser, M.; Schemper, M.; Frisch, H. Alterations in nocturnal serum melatonin levels in humans with growth and aging. *J. Clin. Endocrinol. Metab.* 1988, 66, 648-652. (c) Reiter, R. J. The pineal gland and melatonin in relation to aging: a summary of the theories and of the data. *Exp. Gerontol.* 1995, 30, 199-212.
- (12) (a) Dollins, A. B.; Zhdanova, I. V.; Wurtman, R. J.; Lynch, H. J.; Deng, M. H. Effect of inducing nocturnal serum melatonin concentrations in daytime on sleep, mood, body temperature, and performance. *Proc. Natl. Acad. Sci. U.S.A.* 1994, 91, 1824-1828. (b) Sack, R. L.; Lewy, A. J.; Parrott, K.; Singer, C. M.; McArthur, A. J.; Blood, M. L.; Bauer, V. K. Melatonin analogues and circadian sleep disorders. *Eur. J. Med. Chem.* 1995, 30, 661s-669s. (c) Zhdanova, I. V.; Wurtman, R. J.; Lynch, H. J.; Ives, J. R.; Dollins, A. B.; Morabito, C.; Matheson, J. K.; Schomer, D. L. Sleep-inducing effects of low doses of melatonin ingested in the evening. *Clin. Pharmacol. Ther. (St. Louis)* 1995, 57, 552-558. (d) Attenburrow, M. E. J.; Cowen, P. J.; Sharpley, A. L. Low dose melatonin improves sleep in healthy middle-aged subjects. *Psychopharmacology (Berlin)* 1996, 126, 179-181. (e) Garfinkel, D.; Laudon, M.; Zisapel, N. Improvement of sleep quality by controlled-release melatonin in benzodiazepine-treated elderly insomniacs. *Arch. Gerontol. Geriatr.* 1997, 24, 223-231.
- (13) (a) Liebmann, P. M.; Weller, A.; Felsner, P.; Hofer, D.; Schauenstein, K. Melatonin and the immune system. *Int. Arch. Allergy Immunol.* 1997, 112, 203-211. (b) Reiter, R. J.; Maestroni, G. J. M. Melatonin in relation to the antioxidative defense and immune systems: possible implications for cell and organ transplantation. *J. Mol. Med.* 1999, 77, 30-39.
- (14) Hill, S. M.; Blask, D. E. Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. *Cancer Res.* 1988, 48, 6121-6126.
- (15) (a) Claustrat, B.; Le Bars, D.; Brun, J.; Thivolle, P.; Mallo, C.; Arendt, J.; Chazot, G. Plasma and brain pharmacokinetic studies in humans after intravenous administration of cold or ¹⁴C labeled melatonin. *Adv. Pineal Res.* 1989, 3, 305-310. (b) Le Bars, D.; Thivolle, P.; Vitte, P. A.; Bojkowski, C.; Chazot, G.; Arendt, J.; Frackowiak, R. S. J.; Claustrat, B. PET and plasma pharmacokinetic studies after bolus intravenous administration of [¹⁴C]-melatonin in humans. *Nucl. Med. Biol.* 1991, 18, 357-362.
- (16) Ebisawa, T.; Karne, S.; Lerner, M. R.; Reppert, S. M. Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. *Proc. Natl. Acad. Sci. U.S.A.* 1994, 91, 6133-6137.
- (17) (a) Reppert, S. M.; Godson, C.; Mahle, C. D.; Weaver, D. R.; Slougenhaupt, S. A.; Cusella, J. F. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel_{1b} melatonin receptor. *Proc. Natl. Acad. Sci. U.S.A.* 1995, 92, 8734-8738. (b) Liu, C.; Weaver, D. R.; Jin, X.; Shearman, L. P.; Pieschl, R. L.; Gribkoff, V. K.; Reppert, S. M. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* 1997, 19, 91-102. (c) Dubocovich, M. L.; Yun, K.; Al-ghoul, W. M.; Benloucif, S.; Masana, M. I. Selective MT₂ melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. *FASEB J.* 1998, 12, 1212-1220.
- (18) (a) Paul, P.; Lahaye, C.; Delagrè, P.; Nicolas, J.-P.; Canet, E.; Boutin, J. A. Characterization of 2-[¹²⁵I]iodomelatonin binding sites in Syrian hamster peripheral organs. *J. Pharmacol. Exp. Ther.* 1998, 280, 334-340. (b) Nojean, O.; Ferro, M.; Cogé, F.; Beauverger, P.; Henlin, J.-M.; Lefoulon, F.; Fauchère, J.-L.; Delagrè, P.; Canet, E.; Boutin, J. A. Identification of the melatonin-binding site MT₂ as the quinone reductase 2. *J. Biol. Chem.* 2000, 275, 31311-31317.
- (19) (a) Fourmaïntraux, E.; Depreux, P.; Lesieur, D.; Guardolola-Lemaître, B.; Bennejean, C.; Delagrè, P.; Howell, H. E. Tetrahydronaphthalene derivatives as new agonist and antagonist ligands for melatonin receptors. *Bioorg. Med. Chem.* 1998, 6, 9-13. (b) Kloubert, S.; Mathé-Aillanmat, M.; Andrieux, J.; Siesic, S.; Langlois, M. Synthesis of benzocycloalkane derivatives as new conformationally restricted ligands for melatonin receptors. *Bioorg. Med. Chem. Lett.* 1998, 8, 3325-3330.
- (20) (a) Ledere, V.; Depreux, P.; Lesieur, D.; Calgnard, D. H.; Renard, P.; Delagrè, P.; Guardolola-Lemaître, B.; Morgan, P. Synthesis and biological activity of conformationally restricted tricyclic analogues of the hormone melatonin. *Bioorg. Med. Chem. Lett.* 1996, 6, 1071-1076. (b) Spadoni, G.; Balsamini, C.; Diamantini, G.; Giacomini, B. D.; Tarzia, G. Conformationally restrained melatonin analogues: synthesis, binding affinity for the mel-

- tonin receptor, evaluation of the biological activity, and molecular modeling study. *J. Med. Chem.* 1997, 40, 1990–2002. (c) Beresford, I. M.; Browning, C.; Starkoy, S. J.; Brown, J.; Foord, S. M.; Coughlan, J.; North, P. C.; Dubocovich, M. L.; Hagan, R. M. GR196429: A nonindolic agonist at high-affinity melatonin receptors. *J. Pharmacol. Exp. Ther.* 1998, 285, 1230–1245. (d) Davies, D. J.; Garratt, P. J.; Tocher, D. A.; Vonhoff, S. Mapping the melatonin receptor. 5. Melatonin, Agonists and antagonists derived from tetrahydrocyclopent(b)indoles, tetrahydrocarbazoles and hexahydrocyclopent(b)indoles. *J. Med. Chem.* 1998, 41, 451–467. (e) Marot, C.; Chavatte, P.; Morin-Alory, L.; Vlaud, M. C.; Cullaumet, G.; Renard, P.; Lesieur, D.; Michel, A. Pharmacophoric search and 3D-QSAR comparative molecular field analysis studies on agonists of melatonin sheep receptors. *J. Med. Chem.* 1998, 41, 4453–4465. (f) Jellmann, C.; Mathe-Aillainmat, M.; Andrieux, J.; Kloubert, S.; Boutin, J. A.; Nicolas, J.-P.; Bennejean, C.; Delagrangé, P.; Langlois, M. Synthesis of phenylene and acenaphthene derivatives as new conformationally restricted ligands for melatonin receptors. *J. Med. Chem.* 2000, 43, 4051–4062. (g) Poel, H. V.; Guillaumet, G.; Vlaud-Massuard, M.-C. Synthesis of 6,7,8,9-tetrahydropyrido[2,3-*b*]indolizine and 3,4-dihydro-2*H*-pyrido[2',3':4,5]pyrrolo[2,1-*b*][1,3]oxazine derivatives as new melatonin receptor ligands. *Tetrahedron Lett.* 2002, 43, 1205–1208.
- (21) Paquette, L. A. *Organic Reactions*; John Wiley & Sons: New York, 1997; pp 1–330.
- (22) Monto, A. P.; Marona-Lewicka, D.; Cozzi, N. V.; Nichols, D. E. Synthesis and pharmacological examination of benzofuran, indan, and tetralin analogues of 3,4-(methylenedioxy)amphetamine. *J. Med. Chem.* 1993, 36, 3700–3706.
- (23) House, H. O.; Hudson, C. B. Perhydroindan derivatives. XII. 6-Methoxyindanone and its derivatives. *J. Org. Chem.* 1970, 35, 647–651.
- (24) (a) Wittig, G.; Schölkopf, U. Triphenylphosphinemethylene as an olefin-forming reagent. I. *Chem. Ber.* 1954, 87, 1318–1330. (b) Wadsworth, W. S., Jr.; Emmons, W. D. The utility of phosphonate carbanions in olefin synthesis. *J. Am. Chem. Soc.* 1961, 83, 1733–1738.
- (25) Lindberg, U. H.; Nylen, B.; Akerman, B. Potential local anaesthetics. I. Basic *N*-(α -cydoalkylbenzyl)acylamides and some related compounds. *Acta Pharm. Suec.* 1968, 5, 420–440.
- (26) (a) Kubo, I.; Ochi, M.; Shibata, K.; Hanke, F. J.; Nakatsu, T.; Tan, K.-S.; Taniguchi, M.; Kamikawa, T.; Yamagawa, Y.; Ari-zuka, M.; Wood, W. F. Effect of a marine algal constituent on the growth of lettuce and rice seedlings. *J. Nat. Prod.* 1990, 53, 50–56. (b) Ford, P. W.; Narbut, M. R.; Bell, J.; Davidson, B. S. Synthesis and structural properties of the benzopentathiepins varaceln and isolissocelnitoxin A. *J. Org. Chem.* 1994, 59, 5955–5960.
- (27) (a) De Silva, S. O.; Ahmad, I.; Snleekus, V. A convergent route to phthalideloquinoline alkaloids via directed metalation of tertiary benzamides. *Can. J. Chem.* 1979, 57, 1598–1605. (b) Bellamy, F. D.; Chazan, J. B.; Ou, K. Synthesis of derivatives structurally related to glazlovine. *Tetrahedron* 1983, 39, 2803–2806.
- (28) Castillo, P.; Rodriguez-Ubils, J. C. A high-yield method for the methylation of α -dihydroxyaromatic compounds: synthesis of methylenedioxycompounds. *Synthesis* 1986, 839–840.
- (29) Stewart, J. J. Optimization of parameters for semiempirical methods. *J. Comput. Chem.* 1989, 10, 209–220.
- (30) Vanecok, J.; Vollrath, L. Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. *Brain Res.* 1988, 505, 157–159.
- (31) Polczewski, K.; Kumasaka, T.; Horl, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Trung, I. L.; Teller, D. C.; Okada, T.; Sankamp, R. E.; Yamamoto, M.; Miyano, M. Crystal Structure of Rhodopsin: A G Protein-coupled Receptor. *Science* 2000, 289, 739–745.
- (32) Kokkola, T.; Watson, M.-A.; White, J.; Dowell, S.; Foord, S. M.; Laitinen, J. T. Mutagenesis of Human Mel_{1a} Melatonin Receptor Expressed in Yeast Reveals Domains Important for Receptor Function. *Biochem. Biophys. Res. Commun.* 1998, 249, 531–536.
- (33) Conway, S.; Drew, J. E.; Mowat, E. S.; Barrett, P.; Delagrangé, P.; Morvan, P. J. Chimeric Melatonin m₁ and Melatonin-related Receptors. *J. Biol. Chem.* 2000, 275, 20602–20609.
- (34) De Silva, S. O.; Ahmad, I.; Snleekus, V. A convergent route to phthalideloquinoline alkaloids via directed metalation of tertiary benzamides. *Can. J. Chem.* 1979, 57, 1598–1605.
- (35) Rivkees, S. A.; Cassone, V. M.; Weaver, D. R.; Reppert, S. M. Melatonin receptors in chick brain: characterization and localization. *Endocrinology* 1989, 125, 363–368.
- (36) Niles, L. P.; Pickering, D. S.; Sayer, B. C. HPLC-purified 2-[¹²⁵I]-iodomelatonin labels multiple binding sites in hamster brain. *Biochem. Biophys. Res. Commun.* 1981, 147, 949–950.
- (37) Snider, R. S.; Nemer, W. T. *A Stereotaxic Atlas of the Cat Brain*; University of Chicago Press: Chicago, IL, 1961.

Experiment

Pharmacology. Preparation of CHO Membranes for Receptor Binding Assays. cDNA encoding the human MT₁ gene was introduced into CHO cells. A cell line stably expressing the MT₁ receptor (CHO-hMelR7) was selected and cultured in Eagle's Minimum Essential Medium- α (MEM- α) supplemented with 10% dialyzed fetal bovine serum (dFBS) in a 5% CO₂/95% air atmosphere. Cells were harvested at confluence in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution containing 5 mM EDTA and collected by centrifugation. Cells were homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25 °C), washed twice, pelleted, and stored at -30 °C until binding assays.

Affinity for MT₁ Melatonin Receptor. The procedure of this binding assay was based on the method of Rivkees et al. with minor modifications. The frozen homogenate was thawed, suspended in ice-cold 50 mM Tris-HCl buffer (pH 7.7 at 25 °C), and used for the binding assay. For the assay using CHO-hMelR7 homogenate, 10 μ L of DMSO solution of a test compound was mixed with homogenate and 40 pM (MT₁) of 2-[¹²⁵I]iodomelatonin in a total volume of 1 mL and incubated at 25 °C for 150 min. The reaction was terminated by addition of 3 mL of ice-cold buffer followed by vacuum filtration on Whatman GF/B. The filter was further washed twice with 3 mL of ice-cold buffer, and the radioactivity was measured by a γ -counter. Nonspecific binding was defined in the presence of 10 μ M melatonin. The 50% inhibitory concentration (IC₅₀) was calculated using log-probit analysis. The dissociation constant of the compound for the receptor (K_i) was calculated using the following equation:

$$K_i = IC_{50} / (1 + L / K_d)$$

where L and K_d represent the concentration and the affinity constant of 2-[¹²⁵I]iodomelatonin in the binding assay, respectively.