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Axial chirality and affinity at the $GABA_A$ receptor of pyrimido[1,2-*a*][1,4]benzodiazepines and related compounds

Shoukou Lee [b], Tomonori Kamide [b], Hidetsugu Tabata [a], Hideyo Takahashi [a], Motoo Shiro [c], Hideaki Natsugari [a,*]

[a] *School of Pharmaceutical Science, Teikyo University, 1091-1 Sagamiko, Sagamihara, Kanagawa 229-0195, Japan*
[b] *Graduate School of Pharmaceutical Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[c] *X-Ray Research Laboratory, Rigaku Co. Ltd, 3-9-12 Matsubara, Akishima, Tokyo 196-8666, Japan*

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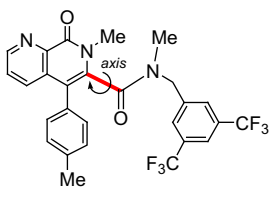
ABSTRACT

The pyrimido[1,2-*a*][1,4]benzodiazepines (**1a–c**) and the 8-membered analogues (diazocines **2a** and **2b**) were separated into their atropisomers with HPLC on a chiral column. High stereochemical stability was observed in the atropisomer of the 8-membered derivatives (**2a** and **2b**), and the 1,4-benzodiazepine (**1c**) with 2′-chloro at the pendant phenyl showed a lower energy barrier for the conversion between the atropisomers compared with that with the unsubstituted pendant phenyl (**1a**). The a*R* isomer of **1a–c** was revealed to be the eutomer in $GABA_A$ receptor binding, and the eutomer **1c-*R*** showed extremely potent activity with an IC_{50} value of 1.5 nM.

1. Introduction

Recently, we have been interested in axial chirality and its relationship with biological activities.[1] Since axial chirality is caused by a conformational change, it may occur in many organic molecules in various forms. It should be noted that if such molecules have biological activities, chirality will be recognized by target molecules, such as receptors and enzymes. For example, the recently discovered tachykinin NK_1 antagonist, TAK-622,[1a] possesses an sp^2–sp^2 axial chirality based on the benzene-amide axis (Fig. 1). Thus, the compound is racemic, and the enantiomers were separated with HPLC on a chiral column, among which the eutomer (that is, the active form) has been revealed to be a*R*.[1b,1c]

1,4-Benzodiazepines as represented by diazepam are the drugs of the first choice for sedation and treatment of anxiety, sleep disorders, etc. and act on the central nervous system (CNS). During the early 1970s, the 1,4-benzodiazepines with a tricyclic structure were extensively researched worldwide, since these compounds showed remarkable activity profiles, especially in vivo. As a result, the tricyclic derivatives with a triazole ring, such as estazolam[2] and triazolam (Fig. 3),[3] were introduced on the market as hypnotics. One of the authors (HN) was involved in a project attempting to discover the tricyclic 1,4-benzodiazepines, and synthesized the tricyclic 1,4-benzodiazepines with a pyrimidone ring (pyrimido[1,2-*a*][1,4]benzodiazepines) (general structure: **A**) (Fig. 2),[4] some of which showed excellent activity in the CNS. The study of those compounds, however, was halted, because estazolam was successfully developed as a hypnotic, and compounds **A** received little attention thereafter. Recently, we have carefully reexamined the structure of **A** and realized that it has a sterically hindered sp^2–sp^2 axis between the pyrimidone and benzene rings and should thus occur as racemates of the a*R* (**A–*R***) and a*S* (**A–*S***) isomers, as shown in Figure 2). Thus, we reexamined the compound **A** using current chemical techniques, that is, high-performance liquid chromatography (HPLC) using a chiral column. This paper deals with



chirality at axis	IC_{50} (nM)
a*R*+a*S*	0.34
a*R*	0.24
a*S*	1.4

TAK-622 (a*R*+a*S*)

Figure 1. TAK-622 as NK_1 antagonist.

* Corresponding author. Tel./fax: +81 426 85 3729.
E-mail address: natsu@pharm.teikyo-u.ac.jp (H. Natsugari).

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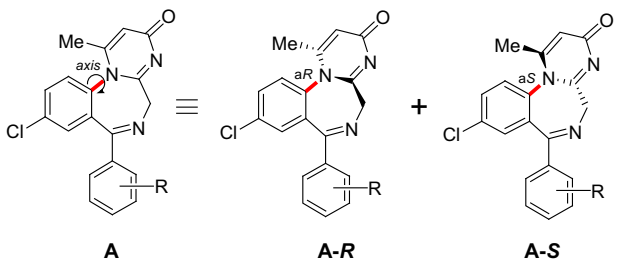

Figure 2. Pyrimido[1,2-*a*][1,4]benzodiazepine (**A**) and the atropisomers (**A-*R*** and **A-*S***).

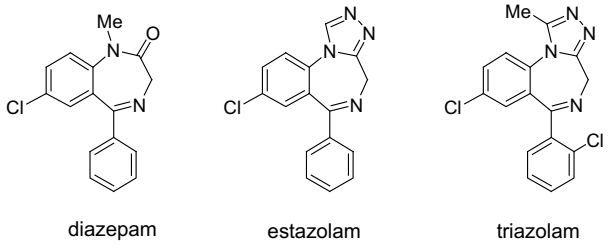

Figure 3. 1,4-Benzodiazepines clinically used.

the separation of the atropisomers of the pyrimido-1,4-benzodiazepines (**1a–c**) and related compounds (8-membered ring compounds **2a** and **2b**) and their affinity for the $GABA_A$ receptor.

2. Results and discussion

The tricyclic compounds with pyrimidone rings analyzed in this paper are shown in Fig. 4. The pyrimido[1,2-*a*][1,4]benzodiazepines **1a** and **1c** are the 3-one derivatives, and **1b** is the regioisomer (1-one form). The 8-membered analogue (pyrimido[1,2-*a*][1,5]benzodiazocine) **2a** is the 3-one derivative, and **2b** is the regioisomer (1-one form). These compounds are those synthesized previously,[4] which were prepared starting from 2-amino-7-chloro-5-phenyl-3*H*-1,4-benzodiazepine, 2-amino-7-chloro-5-(2-chlorophenyl)-3*H*-1,4-benzodiazepine and 2-amino-8-chloro-6-phenyl-3,4-dihydro-1,5-benzodiazocine by acylation with diketene followed by dehydration.[4]

2.1. Affinity at the $GABA_A$ receptor of the original compounds 1 and 2

In 1977, the target molecule of 1,4-benzodiazepines was clarified and identified to be the $GABA_A$ receptor complex.[5] Thus, during the early 1970s, the biological activity in the CNS was evaluated using in vivo (mice, rats) rather than in vitro systems. The pyrimido[1,2-*a*][1,4]benzodiazepines (**1a–c**) showed excellent in vivo activity, while the 8-membered analogues (pyrimido[1,2-*a*][1,5]benzodiazocines) (**2a** and **2b**) did not. In the present study, the in vitro affinity at the $GABA_A$ receptor of the original compounds, **1a–c**, **2a**, and **2b** (Fig. 4) was first evaluated, in which high affinity was observed in the 1,4-benzodiazepines **1a–c** (Table 1). The pyrimidin-3-one derivatives (**1a, 1c**) exhibited greater potency than the regio-isomer (1-one form) (**1b**). Especially noted was the superior potency of **1c** with a 2′-chloro group at the pendant phenyl (IC_{50} 4 nM), which is the same level of activity as that of triazolam. Corresponding well to the in vivo potency, the 8-membered ring compounds **2a** and **2b** did not show any significant affinity in vitro even at 10 μM.

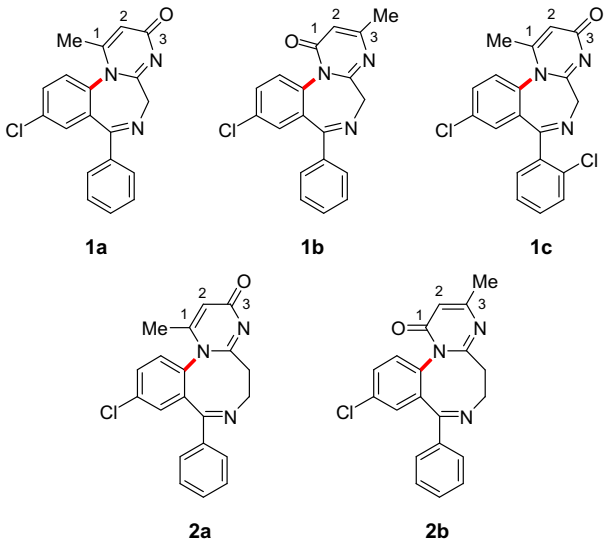

Figure 4. Tricyclic compounds with pyrimidone rings analyzed in this paper.

Table 1
Affinity at the $GABA_A$ receptor of 1,4-benzodiazepines and the related compounds[a]

Compound	IC_{50} (nM)
1a	175
1b	394
1c	4.35
2a	24%[b]
2b	−18%[b]
Estazolam	46.6
Triazolam	1.54
Diazepam	21.2

[a] Benzodiazepine (central) receptor (see Section 4.5).
[b] Inhibition % at 10 μM.

2.2. Separation of the atropisomers of compounds 1 and 2 using HPLC on a chiral column

Compounds **1a–c**, **2a** and **2b** were analyzed using HPLC on a chiral column (CHIRALPAK AD-H) at 18 °C. As expected, all compounds were observed as two separated peaks on HPLC, indicating that they exist as racemates of the atropisomers. The a*R*- and a*S*-

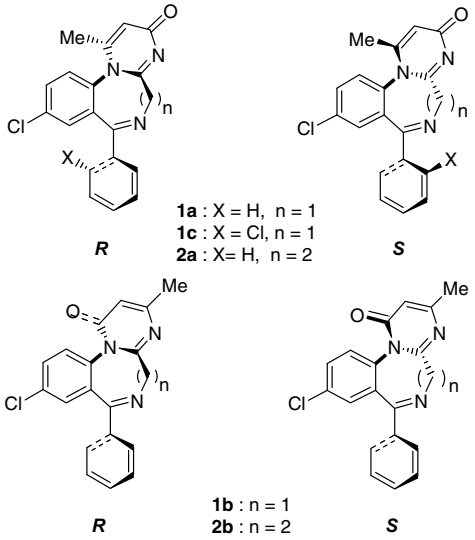

Figure 5. Atropisomers of **1a–c**, and **2a**,**b**.

atropisomers of **1a–c**, **2a** and **2b** were isolated using preparative HPLC (Fig. 5). The separated atropisomers have opposite $[\alpha]_D$ values and CD spectral patterns (Table 2).

Table 2
Physicochemical properties of the separated atropisomers

Compound	HPLC[a] retention time (min)	$[\alpha]^{20}_D$[b]	CD nm (θ)
1a–*R*	10.33	−52.4[c]	306 (−45328), 275 (+197011), 224 (−198086)[d]
1a–*S*	7.26	+33.2[e]	306 (+40529), 275 (−172525), 224 (+177198)[f]
1b–*R*	8.44	−159.2[e]	303 (−32035), 272 (+69467), 251 (−40336), 219 (+227966)[f]
1b–*S*	13.65	+123.6[g]	304 (+30229), 272 (−67727), 252 (+39689), 219 (−217712)[h]
1c–*R*	10.76	−226.5[g]	306 (−26238), 274 (+167771), 251 (−53550), 224 (−208302)[f]
1c–*S*	12.43	+180.6[i]	306 (+26400), 274 (−167100), 251 (+56000), 224 (+221000)[f]
2a–*R*	6.93	−41.0[i]	290 (−5589), 250 (+151703), 220 (−113755)[f]
2a–*S*	6.21	+33.1[g]	288 (+1944), 251 (−166928), 219 (+126286)[h]
2b–*R*	13.97	−2.5[c]	273 (−77994), 241 (+43932), 218 (−191396)[d]
2b–*S*	7.30	+2.8[e]	272 (+78154), 243 (−49787), 218 (+201957)[f]

[a] As for separation conditions, see Section 4.4.
[b] Measured in MeOH.
[c] *c* (concentration) = 0.023 g/mL.
[d] *c* = 0.13 mmol/L.
[e] *c* = 0.024 g/mL.
[f] *c* = 0.14 mmol/L.
[g] *c* = 0.026 g/mL.
[h] *c* = 0.15 mmol/L.
[i] *c* = 0.025 g/mL.

Table 3
Affinity at the $GABA_A$ receptor of atropisomers of **1a–c**[a]

Compound	IC50 (nM)
1a–*R*	46.0
1a–*S*	2320
1b–*R*	93.6
1b–*S*	17%[b]
1c–*R*	1.50
1c–*S*	8.77

[a] Benzodiazepine (central) receptor (see Section 4.5).
[b] Inhibition% at 10 μM.

2.3. Stereochemistry of the atropisomers and their affinity at the $GABA_A$ receptor

After obtaining the atropisomerically pure isomers of **1a–c**, **2a** and **2b** (Fig. 5), we then examined their stereochemistry and affinity at the $GABA_A$ receptor (Table 3).

The atropisomers of **1a** (**1a–*R*** and **1a–*S***) exhibited about a 50-fold difference in the affinities at the receptor; one isomer had strong potency with an IC_{50} value of 46 nM, and the other had an IC_{50} value of 2320 nM. The results clearly indicate that the receptor recognizes the stereochemistry of the axial chirality. Fortunately, the single crystal for the X-ray crystal structure analysis of the eutomer was obtained and subjected to analysis. Thus, based on the Flack parameter the absolute configuration of the eutomer was unambiguously determined to be *aR* (i.e., **1a–*R***) (Fig. 6, left).

The absolute stereochemistry of the atropisomers of **1b**, **1c**, **2a**, and **2b** was assigned by comparing with the data obtained for **1a–*R***. That is, as for the atropisomers of the diazepine derivatives **1b** and **1c**, the isomers with higher binding potency (Table 3) were assigned to be a*R*, which is well supported by the $[\alpha]_D$ data and CD patterns (Table 2). As for the atropisomers of the diazocine derivatives **2a** and **2b**, the absolute stereochemistry was deduced by comparing the $[\alpha]_D$ and CD data with those of the diazepine derivatives (Table 2).

The a*R*-isomer of **1c** (**1c–*R***) showed extremely potent activity with an IC_{50} value of 1.5 nM, which is 30-fold more potent than the eutomer of **1a** (**1a–*R***) (46.0 nM). It should be noted that compound **1c** has another chirality around the axis between the diazepine ring and pendant 2′-chlorophenyl, which means that, theoretically, four stereoisomers [two diastereomers, i.e., (aR^*, aR^*) and (aR^*, aS^*)] should be present in **1c**. Interestingly, however, only one diastereomer was observed in the X-ray crystal structure analysis of **1c** (racemate). The relative stereochemistry is shown to be (aR^*, aR^*) with the methyl (at the pyrimidine ring) and chloro groups in the same orientation, as shown in Figure 6 (right).[6] The absolute stereochemistry of the eutomer is deduced to be (a*R*, a*R*) by comparing with the data on the eutomer **1a–*R***, described above. The effect of 2′-chloro group at the pendant phenyl on the activity has been generally shown in the structure–activity relationship studies of 1,4-benzodiazepines to increase the potency; a typical example is that observed in estazolam and triazolam (IC_{50} 46.6 nM vs 1.54 nM) (Table 1). The desirable conformation of the 1,4-benzodiazepines to show high affinity at the receptor may be that with the pendant phenyl perpendicular to the diazepine ring, as shown in Figure 6. The 2′-chloro substituent may play an important role in fixing the conformation, whereas in **1a** the pendant phenyl may not be fixed and freely rotate.

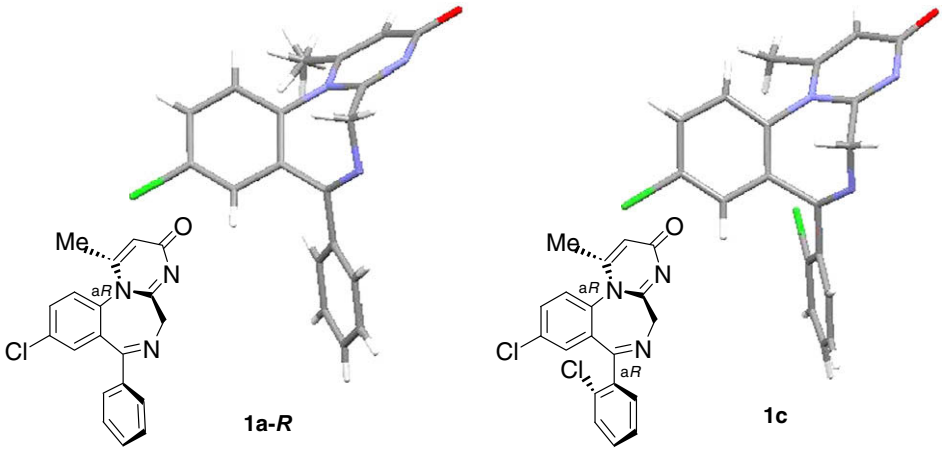

Figure 6. X-ray analyses of **1a–*R*** (eutomer) and **1c** (racemate: only one enantiomer **1c–*R***, presumably the eutomer, is depicted). In the figure of **1a–*R***, a molecule of methanol as the solvate is omitted for clarity and positional disorder at C1-methyl (occurred in a ratio of ca. 4:1) is represented.

Table 4
Stereochemical stability of atropisomers

	Racemization at 37 °C in EtOH	$\Delta G^{\ddagger}$ kcal/mol (kJ/mol)
1a–*R* ⇌ **1a–*S***	ca. 60 h	25.7 (108)
1b–*R* ⇌ **1b–*S***	ca. 60 h	25.8 (108)
1c–*R* ⇌ **1c–*S***	ca. 10 h	24.3 (102)
2a–*R* ⇌ **2a–*S***	[a]	—[b]
2b–*R* ⇌ **2b–*S***	[a]	—[b]

[a] No racemization occurred at 60 °C for 22 h.
[b] Not estimated.

2.4. Stereochemical stability of the atropisomers

Next, the stereochemical stability of these separated atropisomers was examined and it was revealed that all are relatively stable to racemization (Table 4). It is interesting to note that the atropisomers of 8-membered ring derivatives (**2a**, **b**) have extreme stability to racemization compared with the diazepine derivatives (**1a**–**c**) with a 7-membered ring. Compounds **2a–*R*** and **2a–*S*** did not interconvert at all at 60 °C in ethanol even after 22 h, whereas atropisomers of the diazepines **1a**–**c** interconverted at 37 °C in ethanol. The reason for this difference is not apparent, but the stable conformation of the 8-membered ring in **2a** and **2b**, which forms a rigid structure like a cage, may be responsible for the high energy barrier for the conversion of the whole molecule. In the case of the 7-membered ring (diazepine), however, conversion of both the axial chirality and the diazepine ring conformation (flip in a methylene moiety) may operate like a gear to induce relatively ready conversion.

The major difference in stereochemical stability between the diazepines, **1a**, **b** and **1c** is also noteworthy. Although the energy barrier of the conversion at the axis between benzene and pyrimidone appears to be same, the atropisomers of **1a** (**1a–*R*** and **1a–*S***) and **1b** (**1b–*R*** and **1b–*S***) required more than 50 h at 37 °C in ethanol for racemization, whereas those of **1c** (**1c–*R*** and **1c–*S***) racemized in about 10 h. The activation free-energy barrier to rotation ($\Delta G^{\ddagger}$)[7] of the atropisomers of **1a**, **1b** and **1c** determined from the observed data was 25.7, 25.8 and 24.3 kcal/mol, respectively. The lower energy barrier in **1c** than those in **1a** and **1b** is not readily apparent, but it may be caused by the ground-state destabilization in **1c** relative to **1a**, which results from an interaction between the chlorine and imine nitrogen. Also, it may be explained as follows. As shown in the crystal structure (Fig. 6, right), although having two axes, **1c** forms only one diastereomer with relative stereochemistry of (aR^*, aR^*).[6] This means that both axes in **1c** have tendency to move together to adopt that stereochemistry. Thus, the rotation at the axis between the pendant phenyl and diazepine ring in **1c**, which is apparently lower than that of the other axis, may affect the rate of atropisomerism to lower the rotational barrier at the other axis.

3. Conclusions

In summary, the atropisomers of the pyrimido[1,2-*a*][1,4]benzodiazepines (**1a**–**c**) and the 8-membered analogues (**2a** and **2b**) were found to be separated in stable form. High stereochemical stability was observed in the atropisomers of 8-membered ring compounds **2a** and **2b**, and the 1,4-benzodiazepine (**1c**) with 2′-chloro at the pendant phenyl showed a lower energy barrier for the conversion between the atropisomers compared with that with the unsubstituted pendant phenyl (**1a**). The a*R*-isomer of **1a**–**c** was revealed to be the eutomer in $GABA_A$ receptor binding. This study also implies the structure of the active form of triazolam and estazolam. That is, although these tricyclic compounds have been shown to occur as racemates in their single-crystal X-ray analyses (i.e., the space group of triazolam is *C*2/*c*[3b] and that of estazolam is *Pbca*[2c]) and thus may occur as racemates in solution, they presumably adopt the *aR*-conformation at the axis when binding to the receptor.[8]

4. Experimental

4.1. General remarks

Melting points were determined on a Yanagimoto micro melting point apparatus and were uncorrected. ^{1}H NMR spectra were taken on a JEOL JNM-LA400 spectrometer in $CDCl_3$. Chemical shifts were given in ppm with tetramethylsilane as the internal standard and coupling constants (*J*) are given in Hertz (Hz). The following abbreviations are used: s, singlet; d, doublet; m, multiplet. Optical rotations were determined on a JASCO P-1030 digital polarimeter at 20 °C at sodium D-line. Circular dichroism (CD) spectra were obtained at 25 °C with a JASCO J-720 spectropolarimeter.

4.2. Compounds

Compounds **1a**–**c**, **2a** and **2b** were supplied by Takeda Pharmaceutical Co. Ltd, which had been prepared following the reported method[5] and stored as library samples. Estazolam and triazolam were obtained by extraction with $CHCl_3$ of the tablets of Eurodin™ and Halcion™, respectively.

4.3. ^{1}H NMR data of 1a–c, 2a and 2b

The structures of the compounds (**1a–c, 2a** and **2b**) were reexamined by ^{1}H NMR.

4.3.1. 9-Chloro-1-methyl-7-phenylpyrimido[1,2-*a*][1,4]benzodiazepin-3(5*H*)-one (1a)

7.70–7.29 (8H, m), 6.13 (1H, s), 5.08 (1H, d, *J* = 11.9 Hz), 4.03 (1H, d, *J* = 11.9 Hz), 2.11 (3H, s).

4.3.2. 9-Chloro-3-methyl-7-phenylpyrimido[1,2-*a*][1,4]benzodiazepin-1(5*H*)-one (1b)

7.76–7.38 (8H, m), 6.32 (1H, s), 5.07 (1H, d, *J* = 11.9 Hz), 4.06 (1H, d, *J* = 11.9 Hz), 2.28 (3H, s).

4.3.3. 9-Chloro-7-(2-chlorophenyl)-1-methylpyrimido[1,2-*a*][1,4]benzodiazepin-3(5*H*)-one (1c)

7.70–7.17 (7H, m), 6.17 (1H, s), 5.13 (1H, d, *J* = 11.9 Hz), 4.09 (1H, d, *J* = 11.9 Hz), 2.13 (3H, s).

4.3.4. 10-Chloro-5,6-dihydro-1-methyl-8-phenyl-3*H*-pyrimido[1,2-*a*][1,5]benzodiazocin-3-one (2a)

7.65–7.32 (8H, m), 5.95 (1H, s), 4.55 (1H, ddd, *J* = 6.8, 9.3, 13.8 Hz), 3.55 (1H, ddd, *J* = 4.0, 9.7, 13.8 Hz), 3.04 (1H, ddd, *J* = 4.0, 9.3, 13.7 Hz), 2.83 (1H, ddd, *J* = 6.8, 9.7, 13.7 Hz), 1.86 (3H, s).

4.3.5. 10-Chloro-5,6-dihydro-3-methyl-8-phenyl-1*H*-pyrimido[1,2-*a*][1,5]benzodiazocin-1-one (2b)

7.60–7.23 (8H, m), 6.13 (1H, s), 4.43 (1H, ddd, *J* = 4.8, 9.8, 12.7 Hz), 3.47 (1H, ddd, *J* = 4.7, 10.2, 12.7 Hz), 3.17 (1H, ddd, *J* = 4.7, 9.8, 14.7 Hz), 2.95 (1H, ddd, *J* = 4.8, 10.2, 14.7 Hz), 2.21 (3H, s).

4.4. Separation and isolation of atropisomers

The compound (**1a**–**c**, **2a** and **2b**) was separated into the atropisomers by HPLC using CHIRALPAK AD-H (0.46 cmφ × 25 cm) (DAICEL Chemical Industries, Ltd, Japan) under detection at 254 nM using a mixture of hexane/EtOH (1:1) as the eluant at a flow rate of 1.0 mL/min at 18 °C. Retention time in HPLC is shown in Table 2. Each isomer was isolated by preparative HPLC using CHIRALPAK

AD-H (1.0 cmφ × 25 cm, Hexane/EtOH 1:1, flow rate 4.5 mL/min, at 18 °C) as a white powder. The $[\alpha]_D$ data and the CD spectral data are shown in Table 2.

4.5. Affinity at $GABA_A$ receptor

The assay was carried out at MDS Pharma Services. The procedure of the binding assay is as follows. Whole brain (except cerebellum) of male Wister derived rats weighing 175 ± 25 g are used to prepare $GABA_A$ central benzodiazepine membrane receptor in Na–K phosphate buffer pH 7.4. A 5-mg aliquot is incubated with 1 nM [^{3}H]flunitrazepam for 60 min at 25 °C. Non-specific binding is estimated in the presence of 10 μM diazepam. Membranes are filtered and washed. The filters are counted to determine [^{3}H]flunitrazepam specifically bound. The IC_{50} values were determined with inhibition percent obtained at 5-point concentrations (each in duplicate) by a non-linear, least squares regression analysis using Data Analysis Toolbox™ (MDL Information Systems, San Leandro, CA, USA). Diazepam was used as the reference compound. The data are shown in Tables 1 and 3.

4.6. Single-crystal X-ray analyses of 1a–*R* and 1c

4.6.1. X-ray analysis of 1a–*R*

Crystals of **1a–*R*** were grown from MeOH–Et_2O (colorless prisms) as the methanol solvate, mp 135 °C (softened) to 157 °C (decomp.). Data were collected on a diffractometer, Rigaku RAXIS-RAPID using the CrystalStructure10 crystallographic software package [CrystalStructure 3.7.0:Rigaku and Rigaku/MSC (2000–2005)], except for refinement, which was performed using SHELXL-97. Crystallographic data for **1a–*R***: $C_{19}H_{14}ClN_3O_1{\cdot}CH_3OH$, M_r = 367.83, colorless crystal, orthorhombic, space group $P2_12_12_1$, a = 7.4973 (14), b = 11.1986 (2), c = 21.2408 (6) Å, α = 90, β = 90, γ = 90°, V = 1783.4 (6) Å^3, Z = 4, calculated density = 1.370 g/cm^3, absorption coefficient = 2.059 cm^{-1}, T = 298 K, radiation = CuKα (λ = 1.54187 Å), no. of unique reflections = 3231, no. of reflections used for refinement = 2886, R = 0.0519, wR = 0.1585, Flack parameter = 0.01 (2).

4.6.2. X-ray analysis of 1c

Crystals of **1c** were grown from EtOAc–*iso*Pr_2O (colorless prisms), mp 238–239 °C (decomp.) Data were collected on a diffractometer, MacScience M03XHF22 four-circle diffractometer using the MXC data collection package. The structure was solved using the maXus package (MacScience, Japan), which located all the non-hydrogen atoms. Structure refinement was carried out with maXus. All the non-hydrogen atoms are refined anisotropically, and the hydrogen atoms were included in calculated positions using a riding model. Crystallographic data for **1c** $C_{19}H_{13}Cl_2N_3O_1$, M_r = 370.239, colorless crystal, monoclinic, space group $C2/c$, a = 29.110 (13), b = 8.383 (4), c = 46.38 (3) Å, α = 90, β = 109.52 (13), γ = 90°, V = 10668 (10) Å^3, Z = 24, calculated density = 1.213 g/cm^3, absorption coefficient = 0.39 cm^{-1}, T = 298 K, radiation = MoKα (λ = 0.71073 Å), no. of unique reflections = 4868, no. of reflections used for refinement = 2111, R = 0.179, wR = 0.260.

The CIF files of the crystal data for **1a–*R*** and **1c** have been deposited at the Cambridge Crystallographic Data Centre and allocated the Deposition Nos. CCDC 701682 (for **1a–*R***) and 701683 (for **1c**). The data can be obtained free of charge from CCDC via www.ccdc.cam.ac.uk/data_request/cif.

4.7. Determination of the activation free-energy barrier to rotation (ΔG‡)

The $\Delta G^{\ddagger}$ was determined based on the time-dependent conversion rate (% ee) estimated from chiral HPLC analysis of an ethanol solution of the enantiomers (**1a-*R***, **1a-*S***, **1b-*R***, **1b-*S***, **1c-*R*** and **1c-*S***) after allowing to stand at 37 °C. The calculation was carried out according to the procedure reported by Curran et al.[7]

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