Noncompetitive Antagonist-Binding Sites of Rat and Housefly γ-Aminobutyric Acid Receptors Display Different Enantiospecificities for tert-Butyl(isopropyl)bicyclophosphorothionate

Xiu-Lian Ju and Yoshihisa Ozoe*

Department of Life Science and Biotechnology, Shimane University, Matsue, Shimane 690-8504, Japan

Abstract—The enantiomers of 4-*tert*-butyl-3-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide (TBIPPS) were prepared in nine steps from diethyl *tert*-butylmalonate, and their abilities to compete with [^{3}H]1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane (EBOB), a noncompetitive antagonist of ionotropic γ-aminobutyric acid (GABA) receptors, at their binding site were investigated using rat brain and housefly head membranes. The (S)-(−)-isomer of TBIPPS (IC$_{50}$ = 398 nM) was more potent than was the (R)-(+)-isomer of TBIPPS (IC$_{50}$ = 1220 nM) in rat receptors, while the potencies of (S)-TBIPPS (IC$_{50}$ = 104 nM) and (R)-TBIPPS (IC$_{50}$ = 94.4 nM) in housefly receptors were almost the same. The different enantiospecificities of rat and housefly receptors indicate that the three-dimensional structure of the binding site might be different between these receptors. In a region of the rat binding site there might be a steric bulk that interacts less favorably with (R)-TBIPPS than with (S)-TBIPPS, while in the corresponding region of the housefly binding site there might not be such a steric bulk that leads to specificity for these compounds. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Electrophysiological and biochemical studies have demonstrated that bicyclophosphorothionates (2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfides) act as noncompetitive antagonists of ionotropic γ-aminobutyric acid (GABA) receptors in both invertebrate and vertebrate nervous systems.[1–7] Noncompetitive antagonists inhibit the inhibitory action of GABA during synaptic neurotransmission by binding to an allosteric site within GABA receptor-operated chloride channels to exert toxicity in animals.[8] One of the antagonists selective for insect GABA receptors, fipronil, is used as an insecticide.[9]

We recently reported that the introduction of an isopropyl group into the 3-position of bicyclophosphorothionates leads to noncompetitive antagonists with increased affinity and selectivity for housefly (*Musca domestica* L.) GABA receptors versus rat GABA receptors.[10] We noted that *tert*-butylbicyclophosphorothionate (TBPS), which has a 4-*tert*-butyl group but no substituent at the 3-position, was much more selective for rat receptors than

for housefly receptors, as determined by binding assays using the GABA antagonist [^{3}H]1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane (EBOB) (IC$_{50}^{rat}$/IC$_{50}^{fly}$ = 0.0384). The addition of an isopropyl group into the 3-position of TBPS led to *tert*-butyl(isopropyl)bicyclophosphorothionate (TBIPPS), or 4-*tert*-butyl-3-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide, with an IC$_{50}^{rat}$/IC$_{50}^{fly}$ ratio of 13.8. The selectivity value for housefly receptors was thus increased 359-fold by the introduction of an isopropyl group, compared with the selectivity value of TBPS. These findings indicate that the structure of the binding site might be different between rats and houseflies; that is, there might be an ample space that accommodates the 3-isopropyl group of TBIPPS in the housefly binding site but not in the rat binding site.

As TBIPPS is a chiral compound, the separation of the enantiomers may provide more information regarding species differences in the structure of the 3-substituent-interacting area that allows the receptor selectivity. To gain a better understanding of the molecular interaction of TBIPPS with the rat and housefly binding sites, we prepared the enantiomers of TBIPPS, investigated their abilities to compete with [^{3}H]EBOB at the binding site, and report herein the results.

*Corresponding author. Tel.: +81-852-32-6573; fax: +81-852-32-6092; e-mail: ozoe-y@life.shimane-u.ac.jp

Results and Discussion

Chemistry

The enantiomers of TBIPPS were prepared as outlined in Scheme 1. Racemic alcohol **1** was synthesized as previously described.[10] (*S*)-(−)-2-Methoxy-2-trifluoromethylphenylacetic acid (MTPA) reacted via its acid chloride with **1** to give a pair of diastereomeric MTPA esters **2**. Isopropylidene ketal **2** underwent hydrolysis in acetic acid to afford a diastereomeric mixture of diols (*R,S*)- and (*S,S*)-**3**, which were separated using a chiral HPLC column (Sumichiral OA-2000) with hexane:2-propanol (49:1). The absolute stereochemistry of the diastereomers was determined based on their NMR chemical shifts using Mosher's method.[11,12] The proton signal of the *tert*-butyl group of the (*R,S*)-diastereomer was observed at a higher field position than that of the (*S,S*)-diastereomer, because the former is more shielded by the π cloud of the phenyl group of the MTPA moiety (Fig. 1). In contrast, the proton signals of the isopropyl group of the (*R,S*)-diastereomer were centered at a lower field position than those of the (*S,S*)-diastereomer, because the latter is more shielded by the π cloud of the phenyl group of the MTPA moiety. (*R,S*)- and (*S,S*)-**3** were then converted into triols (*R*)-(+)- and (*S*)-(−)-**4**, respectively, using diisobutylaluminum hydride (DIBAL) as a reducing agent. The target compounds ((*R*)-(+)- and (*S*)-(−)-TBIPPS) were finally obtained by the reaction of (*R*)-(+)- and (*S*)-(−)-**4** with thiophosphoryl chloride, respectively. The overall yields of (*R*)- and (*S*)-TBIPPS in nine steps from diethyl *tert*-butylmalonate were 1.4 and 1.3%, respectively.
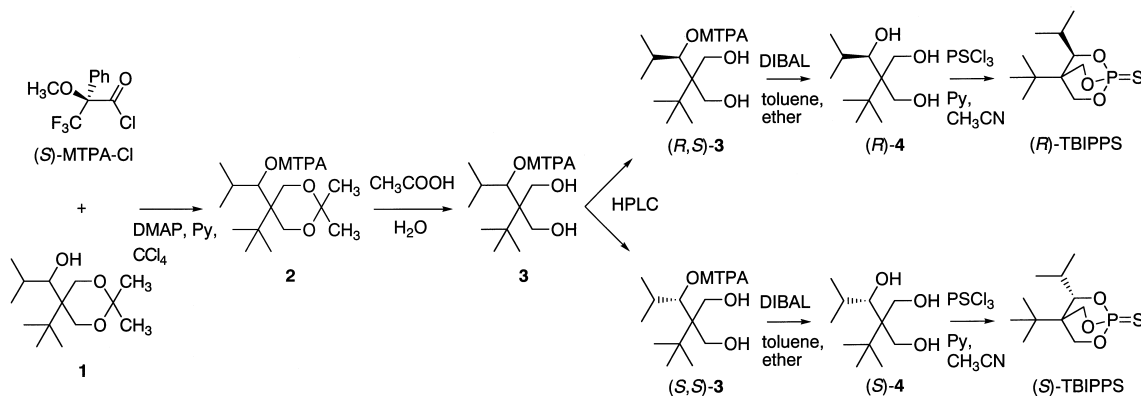
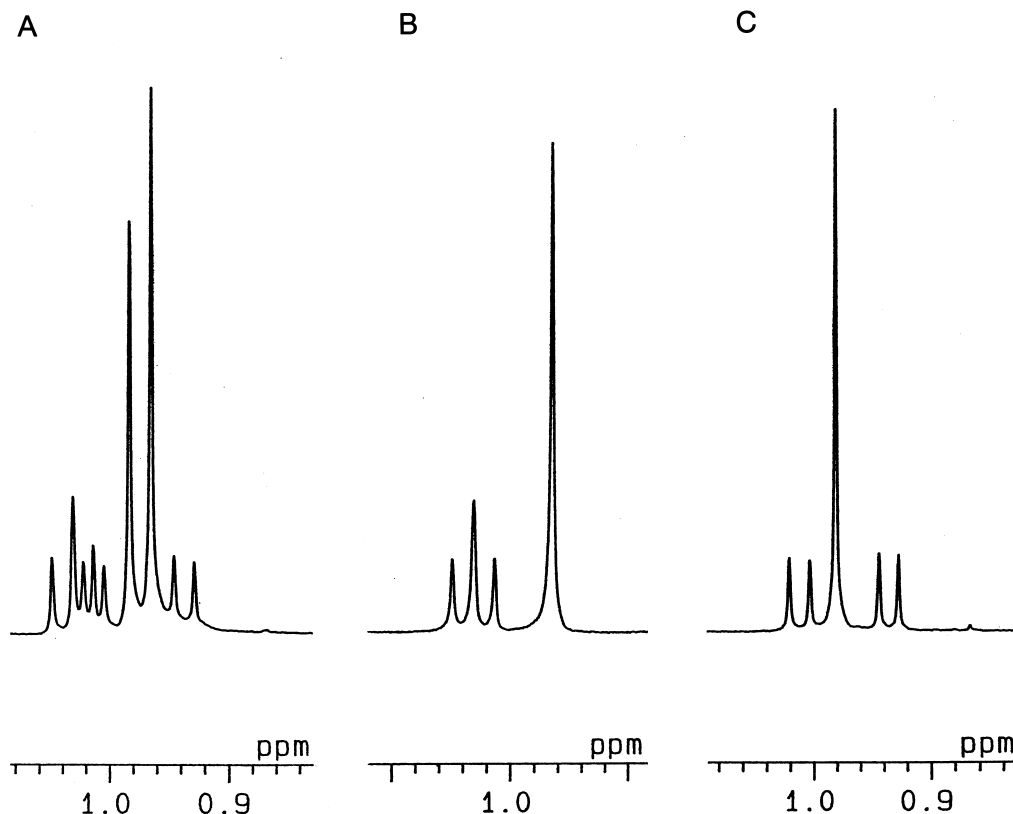**Scheme 1.** Preparation of enantiomers of TBIPPS.

Figure 1. NMR spectra of (A) diastereomeric **3**, (B) (*R,S*)-**3**, and (C) (*S,S*)-**3**.

Receptor binding

We examined (*R*)- and (*S*)-TBIPPS for their potencies to inhibit specific binding of [^{3}H]EBOB, a noncompetitive GABA receptor antagonist, to membranes prepared from rat brains and housefly heads. (*S*)-TBIPPS (IC$_{50}$ = 398 nM) was more potent than (*R*)-TBIPPS (IC$_{50}$ = 1220 nM) in rat brain membranes (Table 1); their concentration–inhibition curves are shown in Figure 2. In contrast, the potencies of (*S*)-TBIPPS (IC$_{50}$ = 104 nM) and (*R*)-TBIPPS (IC$_{50}$ = 94.4 nM) were almost the same in housefly head membranes (Table 1); these concentration–inhibition curves are shown in Figure 3. The previously reported IC$_{50}$ value of racemic TBIPPS was intermediate between those of (*R*)- and (*S*)-TBIPPS in the case of rat receptors, while the IC$_{50}$ value of each enantiomer was not significantly different from that of racemic TBIPPS in the case of housefly receptors (Table 1). The selectivity for housefly GABA receptors estimated in terms of IC$_{50}^{rat}$/IC$_{50}^{fly}$ was >3-fold higher in (*R*)-TBIPPS than in (*S*)-TBIPPS.

The different enantiospecificities of rat and housefly receptors indicate that the three-dimensional structure of the binding site might be different between these

receptors, although the binding site of noncompetitive antagonists is thought to be located in a highly homologous region within GABA receptor-operated chloride channels.[13–17] In a region of the rat binding site there might be a steric bulk that interacts less favorably with (*R*)-TBIPPS than with (*S*)-TBIPPS, while in the corresponding region of the housefly binding site there might not be such a steric bulk exhibiting specificity for these compounds. In particular, the 3-subsitituent-interacting area in housefly receptors appears to be wider than that in rat receptors, because the former can accommodate both enantiomers. These findings support the findings in our previous work that there are structural differences in the noncompetitive antagonist-binding site between insect and mammalian receptors.[10,18,19] Further synthesis of the enantiomers of other bicyclophosphorothionate GABA antagonists and the application of three-dimensional quantitative structure–activity relationship analysis would help us to establish modeling of the binding site and to understand the molecular topography of the antagonist-binding sites of rat and housefly ionotropic GABA receptors.

Experimental

Chemistry

General. Optical rotations were determined with a JASCO DIP-140 digital polarimeter. ^{1}H NMR spectra were obtained in CDCl$_3$ at 400 MHz with a JEOL JNM-A-400 NMR spectrometer, and chemical shifts are reported in parts per million relative to tetramethylsilane as an internal standard. Mass spectra were obtained on a Hitachi M-80B spectrometer. Melting points were determined with a Yanako PM-500D apparatus and are uncorrected. Elemental analyses were performed by Elemental Analysis Center, Faculty of Science, Kyushu

Table 1. Potencies of (*R*)- and (*S*)-TBIPPS in inhibiting [^{3}H]EBOB binding to rat brain and housefly head membranes

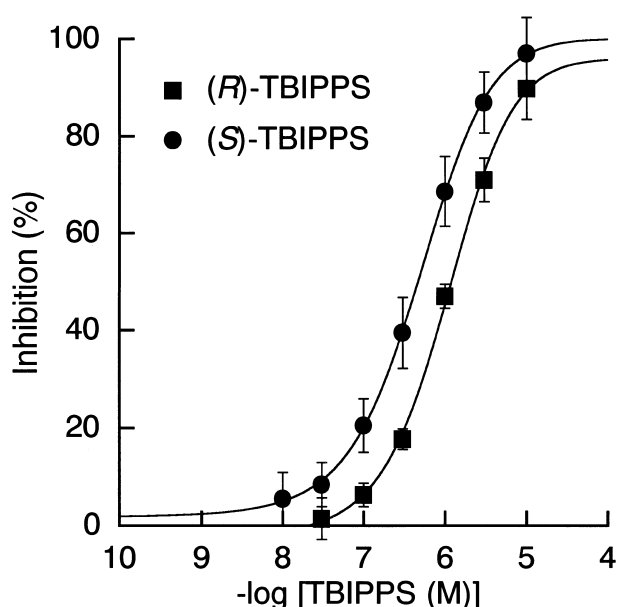| Compound	IC$_{50}$ (nM)		IC$_{50}^{rat}$/IC$_{50}^{fly}$
	Rat	Housefly	
(*R*)-TBIPPS	1220 (990–1500[a])	94.4 (52.8–169)	12.9
(*S*)-TBIPPS	398 (319–497)	104 (64.5–166)	3.83
Racemic TBIPPS[b]	706 (565–883)	51.2 (33.5–78.2)	13.8

[a]95% confidence interval.
[b]Data from ref 10.

Figure 2. Concentration–inhibition curves of (*R*)- and (*S*)-TBIPPS in inhibiting specific [^{3}H]EBOB binding to rat brain membranes.
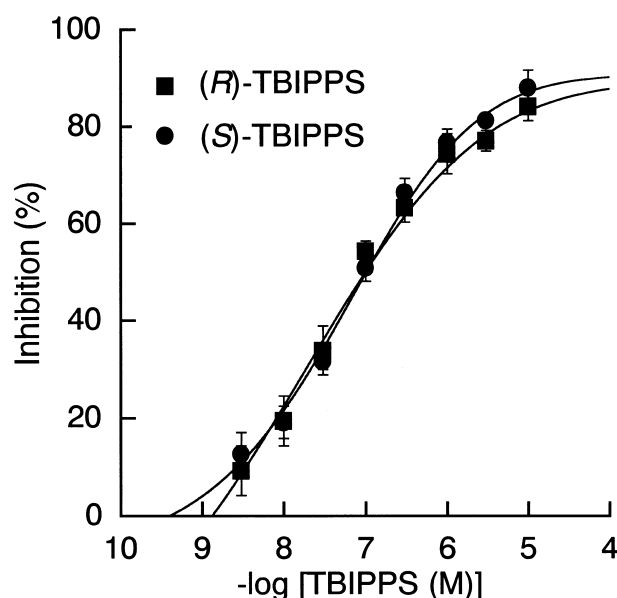

Figure 3. Concentration–inhibition curves of (*R*)- and (*S*)-TBIPPS in inhibiting specific [^{3}H]EBOB binding to housefly head membranes.

University. Reagents were purchased from Wako Pure Chemical Industries, Ltd. [³H]EBOB (1406 GBq/mmol) was purchased from NEN Life Science Products, Inc.

(S)-2-Methoxy-2-trifluoromethylphenylacetyl chloride (MTPA-Cl). A mixture of (S)-(−)-2-methoxy-2-trifluoromethylphenylacetic acid (MTPA) (3.0 g, 12.8 mmol), thionyl chloride (6 mL), and sodium chloride (36 mg) was refluxed for 50 h. After excess thionyl chloride was removed by vacuum evaporation, the residue was distilled under reduced pressure to give 2.7 g (84%) of MTPA-Cl: bp 72–74 °C (3 mm Hg) [lit.[11] bp 54–56 °C (1 mm Hg)].

5-*tert*-Butyl-5-[1-((2S)-2-methoxy-2-trifluoromethylphenylacetoxy)-2-methylpropyl]-2,2-dimethyl-1,3-dioxane (2). 5-*tert*-Butyl-5-(1-hydroxy-2-methylpropyl)-2,2-dimethyl-1,3-dioxane (**1**) was synthesized as previously reported.[10] A mixture of (S)-MTPA-Cl (0.21 g, 0.83 mmol), 5-*tert*-butyl-5-(1-hydroxy-2-methylpropyl)-2,2-dimethyl-1,3-dioxane (0.14 g, 0.57 mmol), 4-dimethylaminopyridine (140 mg), dry pyridine (1 mL), and dry carbon tetrachloride (1 mL) was stirred for 48 h at room temperature. After the addition of water (10 mL), the solution was extracted with ethyl acetate (10 mL×3). The extract was washed with saturated brine, dried (Na₂SO₄), and concentrated under reduced pressure. The residue was purified by chromatography on silica gel with hexane:ethyl acetate (9:1) to give 0.15 g (58%) of a diastereomeric mixture of **2** as a colorless liquid: CIMS (isobutane) m/z 461 (M + 1); ¹H NMR δ 0.84, 0.88 (2s, (CH₃)₃C), 0.96, 0.97, 1.08, 1.09 (4d, $J = 6.8$ Hz, (CH₃)₂CH), 1.31 (s, (CH₃)₂C), 2.52 (m, (CH₃)₂CH), 3.41–3.76 ((CH₂O)₂), 3.49, 3.54 (2CH₃O), 5.22, 5.24 (2d, $J = 2.9, 3.2$ Hz, (CH₃)₂CHCH), 7.41 (m, Ar), 7.58 (m, Ar).

2-*tert*-Butyl-2-[1-((2S)-2-methoxy-2-trifluoromethylphenylacetoxy)-2-methylpropyl]-1,3-propanediol (3). A mixture of **2** (0.20 g, 0.43 mmol), acetic acid (2 mL), and water (1 mL) was heated to 60 °C for 1 h with stirring. After the mixture was cooled, a saturated sodium hydrogen carbonate solution (5 mL) was added to it. The solution was extracted with ether (5 mL×4), and the extract was dried (Na₂SO₄) and concentrated. The residue was purified by chromatography on silica gel with hexane:ethyl acetate (5:1) to give 117 mg (65%) of a diastereomeric mixture of **3** as a colorless liquid: CIMS (isobutane) m/z 421 (M + 1); ¹H NMR δ 0.94 (d, $J = 6.8$ Hz, (CH₃)₂CH)), 0.96 (s, (CH₃)₃C), 0.98 (s, (CH₃)₃C), 1.01 (d, $J = 6.8$ Hz, (CH₃)₂CH), 1.03 (t, $J = 7.1$ Hz, (CH₃)₂CH), 2.34 (m, (CH₃)₂CH), 2.47 (m, OH), 2.56 (t, OH), 2.62 (t, OH), 3.47 (m, CH₃O), 3.55 (dd, CH₂O), 3.57 (m, CH₃O), 3.64 (dd, CH₂O), 3.71 (d, CH₂O), 3.72 (d, CH₂O), 3.78 (dd, CH₂O), 3.80 (dd, CH₂O), 3.91 (dd, CH₂O), 3.92 (dd, CH₂O), 5.29 (dd, $J = 1.7$ Hz, (CH₃)₂CHCH), 5.38 (dd, $J = 2.0$ Hz, (CH₃)₂CHCH), 7.45 (m, Ar), 7.59 (m, Ar).

Separation of the diastereomers of 3. The diastereomers of **3** were separated using a Sumichiral OA-2000 HPLC column (Sumika Chemical Analysis Service, Ltd., 5 μm, 8 mm id×25 cm); mobile phase, hexane:2-propanol (49:1); flow rate, 3 mL/min; detector, 254 nm. 2-*tert*-Butyl-2-[(1R)-1-((2S)-2-methoxy-2-trifluoromethylphe-

nylacetoxy)-2-methylpropyl]-1,3-propanediol ((R,S)-**3**) was obtained as a colorless solid: retention time 22.7 min; mp 75–77 °C; CIMS (isobutane) m/z 421 (M + 1); ¹H NMR δ 0.96 (9H, s, (CH₃)₃C), 1.03 (6H, t, $J = 7.1$ Hz, (CH₃)₂CH), 2.34 (1H, m, (CH₃)₂CH), 2.47 (2H, br, OH), 3.55 (1H, d, CH₂O), 3.57 (3H, s, CH₃O), 3.65 (1H, d, CH₂O), 3.77 (1H, d, CH₂O), 3.91 (1H, d, CH₂O), 5.29 (1H, d, $J = 1.5$ Hz, (CH₃)₂CHCH), 7.44 (3H, m, Ar), 7.58 (2H, m, Ar). 2-*tert*-Butyl-2-[(1S)-1-((2S)-2-methoxy-2-trifluoromethylphenylacetoxy)-2-methylpropyl]-1,3-propanediol ((S,S)-**3**) was obtained as a colorless liquid: retention time 25.6 min; CIMS (isobutane) m/z 421 (M + 1); ¹H NMR δ 0.94 (3H, d, $J = 6.8$ Hz, (CH₃)₂CH), 0.98 (9H, s, (CH₃)₃C), 1.01 (3H, d, $J = 6.8$ Hz, (CH₃)₂CH), 2.33 (1H, m, (CH₃)₂CH), 2.59 (2H, br, OH), 3.47 (3H, s, CH₃O), 3.69 (1H, d, $J = 12.2$ Hz, CH₂O), 3.74 (1H, d, $J = 12.2$ Hz, CH₂O), 3.80 (1H, d, $J = 12.0$ Hz, CH₂O), 3.92 (1H, d, $J = 12.0$ Hz, CH₂O), 5.38 (1H, d, $J = 1.7$ Hz, (CH₃)₂CHCH), 7.46 (3H, m, Ar), 7.60 (2H, m, Ar).

(R)- and (S)-2-*tert*-Butyl-2-hydroxymethyl-4-methyl-1,3-pentanediol (4). A 1.5-M solution of DIBAL in toluene (0.5 mL, 0.75 mmol) was added to a solution of (R,S)-**3** (45 mg, 0.11 mmol) in dry ether (2 mL) with stirring at 0 °C under a nitrogen atmosphere. After stirring at ambient temperature for 2 days, the reaction solution was added to a 10% sulfuric acid solution (5 mL), and the reaction solution was extracted with ether (5 mL×4). The combined ether extract was dried (Na₂SO₄) and concentrated. The residue was purified by chromatography on silica gel with hexane:ethyl acetate (1:1) to give 13.3 mg (59%) of (R)-**4** as a colorless solid: mp 49–50 °C; CIMS (isobutane) m/z 205 (M + 1); ¹H NMR δ 0.98 (9H, s, (CH₃)₃C), 1.07 (6H, 2d, $J = 6.8$ Hz, (CH₃)₂CH), 2.23 (1H, m, (CH₃)₂CH), 3.02 (3H, br, OH), 3.79 (1H, s), 3.85 (1H, d, $J = 11.7$ Hz), 4.03 (2H, s), 4.13 (1H, d, $J = 11.7$ Hz); $[\alpha]_D^{20}$ (c 1.33, ethanol) = + 18.1°. A similar reaction of (S,S)-**3** (41 mg) afforded 13.2 mg (66%) of (S)-**4**: $[\alpha]_D^{20}$ (c 1.32, ethanol) = −17.2°. (S)-**4** gave very similar MS and NMR spectra to those of (R)-**4**.

(R)- and (S)-4-*tert*-Butyl-3-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide (TBIPPS). (R)-TBIPPS (4.5 mg, 26%) was prepared from (R)-**4** (13.3 mg) and thiophosphoryl chloride (11 mg) using a previously reported method:[10] $[\alpha]_D^{20}$ (c 1.20, chloroform) = + 33.3°; HREIMS m/z calcd for C₁₁H₂₁O₃PS 264.0949 (M⁺), found 264.0952. Anal. calcd for C₁₁H₂₁O₃PS: C, 49.98; H, 8.01. Found: C, 50.10, H, 7.83. (S)-TBIPPS (3.7 mg, 22%) was prepared from (S)-**4** (13.2 mg) and thiophosphoryl chloride (11 mg) in the same manner: $[\alpha]_D^{20}$ (c 1.01, chloroform) = −33.6°; HREIMS m/z calcd for C₁₁H₂₁O₃PS 264.0949 (M⁺), found 264.0928. Anal. calcd for C₁₁H₂₁O₃PS: C, 49.98; H, 8.01. Found: C, 49.34; H, 7.69. The ¹H NMR spectra of these two enantiomers were the same as that of racemic TBIPPS.[10]

Binding assays

Rat brain and housefly head P₂ membranes were prepared by a previously reported method.[10] Experiments of [³H]EBOB binding to rat brain and housefly head

X.-L. Ju, Y. Ozoe / Bioorg. Med. Chem. 8 (2000) 2337–2341

2341

membranes were performed in a manner similar to the methods of Cole and Casida[20] and Deng et al.,[21] respectively. In brief, a mixture of DMSO (4 μL), [³H]EBOB (5 nM) in 10 mM phosphate buffer (pH 7.5) containing 300 mM sodium chloride (0.1 mL), and rat brain membranes (125 μg protein/0.9 mL of the same buffer) was used for determination of total binding. The DMSO was replaced with unlabeled 1.25 mM EBOB in DMSO (4 μL) for determination of nonspecific binding, and different concentrations of (R)- and (S)-TBIPPS in DMSO (4 μL) were substituted for DMSO to determine the inhibition caused by the compounds. These mixtures were incubated for 90 min at 37 °C, followed by Brandel M-24 harvester filtration on Whatman GF/B filters and two rinses with 5 mL of ice-cold binding buffer. The filters were subjected to counting in toluene–Methylcellosolve-based scintillation fluid with a Beckman LS 6000 SE scintillation spectrometer. Experiments with housefly head membranes (200 μg protein) were performed using the same procedure except that the incubation time and temperature were 70 min and 22 °C, respectively. The percent inhibition values were calculated. Six to eight concentrations of each test compound were used for determination of the IC_{50} values, which were obtained by the Probit method.

Acknowledgements

References and Notes

1. Bowery, N. G.; Collins, J. F.; Hill, R. G. *Nature* **1976**, *261*, 601.

2. Korenaga, S.; Ito, Y.; Ozoe, Y.; Eto, M. *Comp. Biochem. Physiol.* **1977**, *57C*, 95.

3. Ticku, M. K.; Olsen, R. W. *Neuropharmacology* **1979**, *18*, 315.

4. Squires, R. F.; Casida, J. E.; Richardson, M.; Saederup, E. *Mol. Pharmacol.* **1983**, *23*, 326.

5. Bermudez, I.; Hawkins, C. A.; Taylor, A. M.; Beadle, D. J. *J. Receptor Res.* **1991**, *11*, 221.

6. Dillon, G. H.; Im, W. B.; Carter, D. B.; McKinley, D. D. *Br. J. Pharmacol.* **1995**, *115*, 539.

7. Aydar, E.; Beadle, D. J. *J. Insect Physiol.* **1999**, *45*, 2113.

8. Tulinsky, J.; Gammill, R. B. *Curr. Med. Chem.* **1994**, *3*, 226.

9. Gant, D. B.; Chalmers, A. E.; Wolff, M. A.; Hoffman, H. B.; Bushey, D. F. *Rev. Toxicol.* **1998**, *2*, 147.

10. Ju, X.-L.; Ozoe, Y. *Pestic. Sci.* **1999**, *55*, 971.

11. Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543.

12. Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512.

13. ffrench-Constant, R. H.; Rocheleau, T. A.; Steichen, J. C.; Chalmers, A. E. *Nature* **1993**, *363*, 449.

14. Gurley, D.; Amin, J.; Ross, P. C.; Weiss, D. S.; White, G. *Recept. Channels* **1995**, *3*, 13.

15. Xu, M.; Covey, D. F.; Akabas, M. H. *Biophys. J.* **1995**, *69*, 1858.

16. Perret, P.; Sarda, X.; Wolff, M.; Wu, T.-T.; Bushey, D.; Goeldner, M. *J. Biol. Chem.* **1999**, *274*, 25350.

17. Jursky, F.; Fuchs, K.; Buhr, A.; Tretter, V.; Sigel, E.; Sieghart, W. *J. Neurochem.* **2000**, *74*, 1310.

18. Akamatsu, M.; Ozoe, Y.; Ueno, T.; Fujita, T.; Mochida, K.; Nakamura, T.; Matsumura, F. *Pestic. Sci.* **1997**, *49*, 319.

19. Ozoe, Y.; Akamatsu, M.; Higata, T.; Ikeda, I.; Mochida, K.; Koike, K.; Ohmoto, T.; Nikaido, T. *Bioorg. Med. Chem.* **1998**, *6*, 481.

20. Cole, L. M.; Casida, J. E. *Pestic. Biochem. Physiol.* **1992**, *44*, 1.

21. Deng, Y.; Palmer, C. J.; Casida, J. E. *Pestic. Biochem. Physiol.* **1993**, *47*, 98.