JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

4,5-Substituted 3-Isoxazolols with Insecticidal Activity Act as Competitive Antagonists of Housefly GABA Receptors

Genyan Liu, Fumiyo Ozoe, Kenjiro Furuta, and Yoshihisa Ozoe

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.5b01843 • Publication Date (Web): 29 Jun 2015

Just Accepted

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

4,5-Substituted 3-Isoxazolols with Insecticidal Activity Act as Competitive Antagonists of Housefly GABA Receptors

Genyan Liu,[†] Fumiyo Ozoe,[‡] Kenjiro Furuta,[†,‡] and Yoshihisa Ozoe*[,†,‡]

[†]Division of Bioscience and Biotechnology, The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8553, Japan

[‡]Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504, Japan

ABSTRACT: The insect GABA receptor (GABAR), which is composed of five RDL subunits, represents an important target for insecticides. A series of 4,5-disubstituted 3-isoxazolols, including muscimol analogs, were synthesized and examined for their activities against four splice variants (ac, ad, bc, and bd) of housefly GABARs expressed in *Xenopus* oocytes. Muscimol was a more potent agonist than GABA in all four splice variants, whereas synthesized analogs did not exhibit agonism but rather antagonism in housefly GABARs. The introduction of bicyclic aromatic groups at the 4-position of muscimol and the simultaneous replacement of the aminomethyl group with a carbamoyl group at the 5-position to afford six 4-aryl-5-carbamoyl-3-isoxazolols resulted in compounds that exhibited significantly enhanced antagonism with IC_{50} values in the low micromolar range in the ac variant. The inhibition of GABA-induced currents by 100 μM analogs was approximately 1.5- to 4-fold greater in the ac and bc variants than in the ad and bd variants. 4-(3-Biphenylyl)-5-carbamoyl-3-isoxazolol displayed competitive antagonism, with IC_{50} values of 30, 34, 107, and 96 μM in the ac, bc, ad, and bd variants, respectively, and exhibited moderate insecticidal activity against houseflies, with an LD_{50} value of 5.6 nmol/fly. These findings suggest that these 3-isoxazolol analogs are novel lead compounds for the design and development of insecticides that target the orthosteric site of housefly GABARs.

KEYWORDS: *insect GABA receptors, competitive antagonists, 3-isoxazolols, RDL, two-electrode voltage clamp, insecticidal activity*

INTRODUCTION

γ-Aminobutyric acid (GABA) is a major neurotransmitter in both vertebrates and invertebrates. Ionotropic GABA receptors (GABARs) mediate fast inhibitory synaptic transmission by enhancing membrane permeability to chloride ions in response to GABA, which is released from the presynaptic neuron.[1] In insects, GABARs are predominantly expressed in the central nervous system and play important physiological roles in sleep, olfaction, and learning/memory.[2,3] Because vertebrate and invertebrate GABARs have different pharmacological properties, insect GABARs are an important target for safe insecticides, such as phenylpyrazoles.[2–4]

Ionotropic GABARs are pentameric ligand-gated chloride channels, which belong to the family of Cys-loop receptors. Whereas 19 constitutive subunits are present in mammalian GABARs, RDL is the only subunit that constitutes inhibitory GABARs in insects.[2–4] However, the RDL-encoding gene *Rdl* undergoes alternative splicing of exons 3 and 6 to generate four variants (RDL_{ac}, RDL_{ad}, RDL_{bc}, and RDL_{bd}) in the fruit fly (*Drosophila melanogaster* Meigen) and other insect species.[5] The *Drosophila* variants of GABARs have been reported to exhibit differential agonist sensitivity when expressed in *Xenopus* oocytes.[6–8] The alternative splicing may also increase the pharmacological diversity of insect GABARs. However, the physiology and pharmacology of these variants of GABARs have yet to be characterized.

Efforts focused on GABAR pharmacology recently led to the discovery of two novel chemotypes of insecticides, isoxazolines and benzamides, which act as noncompetitive

antagonists at a unique allosteric site(s) in insect GABARs.[9–13] These insecticides overcome the emerging resistance to conventional GABAR antagonist insecticides (e.g., fipronil) because of their different sites of action. In addition, competitive antagonists, which act at the orthosteric agonist-binding site of GABARs, have the potential to become novel insecticides. In our recent studies, thio-4-PIOL and gabazine analogs (Figure 1) were synthesized and examined for their antagonism of GABARs cloned from three insect species.[14–16] Subsequently, we found that introducing bicyclic aromatic groups into the 4-position of the isothiazole ring of thio-4-PIOL or into the 3-position of the dihydropyridazine ring of gabazine enhances the antagonism of insect GABARs by these analogs. These analogs also showed insecticidal activity, though this activity was moderate. These findings prompted further exploration of potentially novel insecticides acting as competitive GABAR antagonists. Here, we report the synthesis of 4,5-disubstituted 3-isoxazolols (Figure 1) and their differential antagonism of four splice variants of housefly (*Musca domestica* Linnaeus) GABARs.

MATERIALS AND METHODS

Chemistry. Reagents and solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Sigma-Aldrich Co., LLC. (Tokyo, Japan), unless otherwise noted. All air- and moisture-sensitive reactions were performed under an argon atmosphere using oven-dried glassware. Reactions were monitored by thin layer chromatography (TLC Silica gel 60 F254 plates, Merck KGaA,

Darmstadt, Germany) using UV light or a $KMnO_4$ spray reagent. Column chromatography was performed using silica gel (Wakogel® C-200, 75−150 μm, Wako). Melting points were determined using a YANACO MP-500D micro melting point apparatus and are uncorrected. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a JEOL JNM A-400 spectrometer. Chemical shifts are reported in parts per million (ppm) on the δ scale, and coupling constants (J) are in Hertz (Hz). Spin multiplicities are abbreviated as follows: s (singlet), bs (broad singlet), d (doublet), t (triplet), and m (multiplet). Mass spectra (MS) were measured using the positive electrospray ionization mode (ESI) on a Waters XEVO mass spectrometer, and high-resolution mass spectra (HRMS) were obtained using ESI on a Waters SYNAPT G2 spectrometer.

Synthesis of Methyl 3-Hydroxy-5-isoxazolecarboxylate (2).[17] Dimethyl acetylenedicarboxylate (1.4 g, 10.0 mmol) was added to a solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (1.8 g, 12.0 mmol) and *N*-hydroxyurea (760 mg, 10.0 mmol) in methanol (15 mL) at 0 °C under argon. The solution was stirred at 0 °C for 30 min and then at room temperature for 12 h. After the solvent was evaporated, the residue was dissolved in water (25 mL) and acidified to pH 1 with conc. HCl. The product was extracted with Et_2O (3 × 30 mL), and the combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The obtained solid was recrystallized from chloroform to give **2** as a light yellow solid (840 mg, 59% yield). ^1H NMR (400 MHz, DMSO-d_6): δ 11.97 (1H, s, OH), 6.76 (1H, s, Ar-H), 3.87 (3H, s, $COOCH_3$).

Synthesis of 3-Benzyloxy-5-methoxycarbonylisoxazole (3).[18] A mixture of **2** (2.1 g, 15 mmol) and K_2CO_3 (3.1 g, 22.5 mmol) in acetone (30 mL) was heated at 70 °C for 1 h. Benzyl

bromide (3.8 g, 22.5 mmol) was added dropwise, and the mixture was stirred at 50 °C for 12 h. The mixture was filtered, and the filtrate was concentrated under reduced pressure. Water (30 mL) was added to the filtrate, and the product was extracted with Et_2O (3 × 30 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. Column chromatography (hexane/EtOAc 15:1) gave **3** as a colorless oil (3.1 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54–7.28 (5H, m, Ar-H), 6.57 (1H, s, Ar-H), 5.32 (2H, s, OCH_2), 3.95 (3H, s, $COOCH_3$). MS: *m*/*z* 255.9 $[M+Na]^+$.

Synthesis of 3-Benzyloxy-5-carbamoylisoxazole (4). A mixture of **3** (466 mg, 2.0 mmol) and aqueous ammonia (5 mL, 28%) was stirred at room temperature for 12 h. The mixture was concentrated to dryness under reduced pressure. Water (15 mL) was added to the residue, and the mixture was extracted with EtOAc (3 × 15 mL). The combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to afford **4** as a white solid (395 mg, 91% yield). mp 162–164 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48–7.32 (5H, m, Ar-H), 6.59 (1H, s, Ar-H), 6.43 (1H, bs, $CONH_2$), 5.87 (1H, bs, $CONH_2$), 5.31 (2H, s, OCH_2). MS: *m*/*z* 241.1 $[M+Na]^+$.

Synthesis of Muscimol Hydrobromide (5a).[19] Borane in THF (1 M, 5 mL, 5.0 mmol) was slowly added to a solution of **4** (480 mg, 2.2 mmol) in dry THF (15 mL) at 0 °C. The solution was stirred at room temperature for 16 h. After acidification (pH 1) with 4 M HCl, the solution was stirred for 1 h and concentrated under reduced pressure. The residue was suspended in water, and the solution was made basic (pH 10) with 4 M NaOH. The product was extracted with EtOAc (3 × 30 mL); the combined organic phases were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to afford a light yellow oil. The oil

was dissolved in a solution of HBr in AcOH (10 mL, 30%), and the mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated with a subsequent azeotropic treatment of MeOH/toluene (1:1) three times, and the residue was recrystallized (MeOH/Et_2O) to give **5a** as a light brown solid (179 mg, 42% yield). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.25 (1H, bs, OH), 8.45 (3H, bs, NH_2·HBr), 6.18 (1H, s, Ar-H), 4.14 (2H, s, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.14, 165.22, 96.04, 34.29. HRMS: *m*/*z* calcd for $C_4H_7N_2O_2$ $[M–Br]^+$ 115.0508, found 115.0502.

Synthesis of 5-Carbamoyl-3-isoxazolol (6a). Compound **4** (349 mg, 1.6 mmol) was suspended in a solution of HBr in AcOH (10 mL, 30%), and the mixture was stirred at room temperature for 24 h. The reaction mixture was concentrated with a subsequent azeotropic treatment of MeOH/toluene (1:1) three times, and the residue was recrystallized (MeOH/EtOAc) to give **6a** as a white solid (152 mg, 74% yield). mp 236–239 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.61 (1H, bs, OH), 8.15 (1H, bs, $CONH_2$), 7.81 (1H, bs, $CONH_2$), 6.54 (1H, s, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.40, 163.58, 157.39, 97.69. HRMS: *m*/*z* calcd for $C_4H_5N_2O_3$ $[M+H]^+$ 129.0295, found 129.0290.

Synthesis of 3-Benzyloxy-5-carbamoyl-4-iodoisoxazole (7). A mixture of **4** (1.1 g, 5 mmol), $Pd(OAc)_2$ (112 mg, 0.5 mmol), CsOAc (2.3 g, 12 mmol), $NaHCO_3$ (420 mg, 5 mmol), I_2 (3.8 g, 15 mmol), 4 Å molecular sieves (150 mg), and *N*-methylformamide (30 mL) was stirred at 75 °C for 16 h. After cooling, the reaction mixture was added to $Na_2S_2O_4$ (solid) until the color stopped changing, and then it was filtered through Celite®, which was thoroughly washed with EtOAc. The combined organic phases were washed with water twice and then with brine, dried over Na_2SO_4, and evaporated under reduced pressure. Column

chromatography (hexane/EtOAc 10:1) gave **7** as a light yellow solid (980 mg, 57% yield). mp 179–182 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50–7.35 (5H, m, Ar-H), 6.43 (1H, bs, $CONH_2$), 5.94 (1H, bs, $CONH_2$), 5.37 (2H, s, OCH_2). MS: *m*/*z* 366.9 $[M+Na]^+$.

General Procedure for the Synthesis of 4-Aryl-3-benzyloxy-5-carbamoylisoxazole (8b–8g). A mixture of **7** (1 mmol), an arylboronic acid (1.5 mmol), $Pd(PPh_3)_2Cl_2$ (0.08 mmol), DMF (5 mL), and aqueous K_2CO_3 (0.5 mL, 3 M, 1.5 mmol) was stirred at 80 °C for 24 h. After cooling, the reaction mixture was filtered through Celite® and diluted using Et_2O. The organic phase was washed with water and brine, dried over Na_2SO_4, and evaporated under reduced pressure. Column chromatography (hexane/EtOAc) gave **8b**–**g**.

3-Benzyloxy-4-(3-biphenylyl)-5-carbamoylisoxazole (8b). A white solid, yield 82%, mp 145–147 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (1H, s, Ar-H), 7.70–7.26 (13H, m, Ar-H), 6.40 (1H, bs, $CONH_2$), 5.78 (1H, bs, $CONH_2$), 5.42 (2H, s, OCH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.88, 157.95, 156.60, 141.09, 140.78, 135.39, 129.03, 128.83, 128.74, 128.60, 128.56, 128.09, 127.58, 127.37, 127.20, 126.59, 114.21, 72.27. MS: *m*/*z* 371.0 $[M+H]^+$, 393.0 $[M+Na]^+$.

3-Benzyloxy-5-carbamoyl-4-(2-naphthyl)isoxazole (8c). A white solid, yield 69%, mp 138–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14 (1H, s, Ar-H), 7.88–7.73 (4H, m, Ar-H), 7.72–7.26 (7H, m, Ar-H), 6.39 (1H, bs, $CONH_2$), 5.84 (1H, bs, $CONH_2$), 5.43 (2H, s, OCH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.90, 157.95, 156.60, 135.33, 133.21, 132.90, 129.75, 129.58, 128.49, 128.37, 127.90, 127.74, 127.63, 127.26, 126.74, 126.23, 123.56, 114.31, 72.17. MS: *m*/*z* 367.0 $[M+Na]^+$.

3-Benzyloxy-4-(4-biphenylyl)-5-carbamoylisoxazole (8d). A white solid, yield 90%,

mp 164–167 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80–7.28 (14H, m, Ar-H), 6.39 (1H, bs, $CONH_2$), 5.75 (1H, bs, $CONH_2$), 5.42 (2H, s, OCH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.98, 158.03, 156.59, 141.72, 140.66, 135.48, 132.23, 132.13, 130.49, 128.84, 128.67, 128.60, 128.47, 128.08, 127.59, 127.19, 126.95, 125.21, 114.11, 72.34. MS: *m*/*z* 371.0 $[M+H]^+$.

3-Benzyloxy-5-carbamoyl-4-(1-naphthyl)isoxazole (8e). A white solid, yield 77%, mp 154–156 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.95–7.25 (12H, m, Ar-H), 6.05 (1H, bs, $CONH_2$), 5.72 (1H, bs, $CONH_2$), 5.34 (2H, s, OCH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.35, 158.09, 157.37, 135.23, 133.66, 131.57, 129.83, 128.97, 128.52, 128.41, 128.05, 126.56, 126.25, 125.26, 125.19, 123.84, 112.32, 72.02. MS: *m*/*z* 367.1 $[M+Na]^+$.

3-Benzyloxy-5-carbamoyl-4-(6-methoxy-2-naphthyl)isoxazole (8f). A light yellow solid, yield 67%, mp 124–126 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.07 (1H, s, Ar-H), 7.78–7.65 (3H, m, Ar-H), 7.46–7.30 (5H, m, Ar-H), 7.18–7.10 (2H, m, Ar-H), 6.40 (1H, bs, $CONH_2$), 6.06 (1H, bs, $CONH_2$), 5.42 (2H, s, OCH_2), 3.91 (3H, s, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.92, 158.40, 158.21, 156.39, 135.41, 134.54, 129.90, 129.50, 128.55, 128.45, 127.86, 127.79, 126.57, 121.23, 119.11, 114.35, 105.61, 72.12, 55.32. MS: *m*/*z* 397.1 $[M+Na]^+$.

3-Benzyloxy-5-carbamoyl-4-(6-hydroxy-2-naphthyl)isoxazole (8g). A light yellow solid, yield 43%, mp 177–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.73 (1H, s, Ar-OH), 8.11 (1H, bs, $CONH_2$), 7.97 (1H, s, Ar-H), 7.85 (1H, bs, $CONH_2$), 7.75–7.05 (10H, m, Ar-H), 5.41 (2H, s, OCH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.11, 158.67, 158.25, 155.96, 135.67, 134.12, 129.56, 128.60, 128.37, 128.24, 127.93, 127.31, 127.06, 125.49, 120.77, 118.98, 111.59, 108.54, 71.63. MS: *m*/*z* 383.1 $[M+Na]^+$.

Synthesis of 5-Aminomethyl-4-(3-biphenylyl)-3-isoxazolol Hydrobromide (5b). Compound **5b** was prepared from **8b** (185 mg, 0.5 mmol) according to the procedure described for **5a**. Recrystallization (MeOH/Et_2O) gave **5b** as a white solid (69 mg, 40%). mp 220–222 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 7.75–7.30 (9H, m, Ar-H), 4.36 (2H, s, CH_2). ^{13}C NMR (100 MHz, CD_3OD): δ 163.30, 160.88, 143.29, 141.79, 130.50, 129.95, 129.33, 128.72, 128.66, 128.38, 128.07, 128.02, 111.90, 35.40. HRMS: *m*/*z* calcd for $C_{16}H_{15}N_2O_2$ $[M–Br]^+$ 267.1134, found 267.1143.

Synthesis of 5-Aminomethyl-4-(2-naphthyl)-3-isoxazolol Hydrobromide (5c). Compound **5c** was prepared from **8c** (196 mg, 0.57 mmol) according to the procedure described for **5a**. Recrystallization (MeOH/Et_2O) gave **5c** as a brown solid (85 mg, 47%). mp 226–228 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 8.01–7.86 (4H, m, Ar-H), 7.65–7.50 (3H, m, Ar-H), 4.41 (2H, s, CH_2). ^{13}C NMR (100 MHz, CD_3OD): δ 170.22, 160.92, 134.85, 134.37, 129.63, 129.09, 128.77, 127.69, 127.20, 126.21, 112.05, 35.56. HRMS: *m*/*z* calcd for $C_{14}H_{13}N_2O_2$ $[M–Br]^+$ 241.0977, found 241.0989.

Synthesis of 4-(3-Biphenylyl)-5-carbamoyl-3-isoxazolol (6b). Compound **6b** was prepared from **8b** (333 mg, 0.9 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6b** as a white solid (210 mg, 83%). mp 193–195 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.03 (1H, bs, OH), 8.09 (1H, bs, $CONH_2$), 7.90 (1H, s, Ar-H), 7.82 (1H, bs, $CONH_2$), 7.71–7.27 (8H, m, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.62, 158.59, 158.30, 140.04, 139.77, 128.87, 128.42, 128.35, 128.05, 127.43, 126.62, 126.19, 111.31. HRMS: *m*/*z* calcd for $C_{16}H_{13}N_2O_3$ $[M+H]^+$ 281.0921, found 281.0914.

Synthesis of 5-Carbamoyl-4-(2-naphthyl)-3-isoxazolol (6c). Compound **6c** was

prepared from **8c** (189 mg, 0.55 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6c** as a light yellow solid (95 mg, 68%). mp 221–223 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.02 (1H, bs, OH), 8.10 (1H, s, Ar-H), 8.06 (1H, bs, $CONH_2$), 7.95–7.88 (3H, m, Ar-H), 7.79 (1H, bs, $CONH_2$), 7.66 (1H, d, J = 8.3 Hz, Ar-H), 7.58–7.47 (2H, m, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.58, 158.39, 158.28, 132.33, 132.19, 128.35, 127.80, 127.25, 126.92, 126.23, 126.02, 124.96, 111.33. HRMS: m/z calcd for $C_{14}H_{11}N_2O_3$ $[M+H]^+$ 255.0770, found 255.0785.

Synthesis of 4-(4-Biphenylyl)-5-carbamoyl-3-isoxazolol (6d). Compound **6d** was prepared from **8d** (222 mg, 0.6 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6d** as a white solid (112 mg, 67%). mp 278–280 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.03 (1H, bs, OH), 8.09 (1H, bs, $CONH_2$), 7.82 (1H, bs, $CONH_2$), 7.80–7.50 (6H, m, Ar-H), 7.48–7.34 (3H, m, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.61, 158.56, 158.20, 139.65, 139.58, 129.96, 128.86, 127.48, 126.61, 126.54, 126.04, 110.98. HRMS: m/z calcd for $C_{16}H_{12}N_2O_3Na$ $[M+Na]^+$ 303.0740, found 303.0738.

Synthesis of 5-Carbamoyl-4-(1-naphthyl)-3-isoxazolol (6e). Compound **6e** was prepared from **8e** (330 mg, 0.96 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6e** as a white solid (128 mg, 52%). mp 189–191 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.77 (1H, bs, OH), 7.96 (2H, d, J = 7.8 Hz, Ar-H), 7.92 (1H, bs, $CONH_2$), 7.68 (1H, bs, $CONH_2$), 7.59–7.41 (5H, m, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.34, 159.28, 157.69, 133.03, 131.46, 128.66, 128.44, 128.07, 126.08, 125.76, 125.39, 125.15, 110.58. HRMS: m/z calcd for $C_{14}H_{11}N_2O_3$ $[M+H]^+$ 255.0770, found

255.0785.

Synthesis of 5-Carbamoyl-4-(6-methoxy-2-naphthyl)-3-isoxazolol (6f). Compound **6f** was prepared from **8f** (243 mg, 0.65 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6f** as a white solid (115 mg, 62%). mp 242–244 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.99 (1H, bs, OH), 8.03 (2H, bs, $CONH_2$), 7.85–7.70 (4H, m, Ar-H), 7.63 (1H, dd, J = 8.8, 2.0 Hz, Ar-H), 7.32 (1H, d, J = 2.4 Hz, Ar-H), 7.17 (1H, dd, J = 8.8, 2.4 Hz, Ar-H), 3.89 (3H, s, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.72, 158.58, 158.05, 157.71, 133.70, 129.52, 128.31, 127.89, 127.83, 125.99, 122.60, 118.64, 111.58, 105.90, 55.21. HRMS: m/z calcd for $C_{15}H_{12}N_2O_4Na$ $[M+Na]^+$ 307.0695, found 307.0691.

Synthesis of 5-Carbamoyl-4-(6-hydroxy-2-naphthyl)-3-isoxazolol (6g). Compound **6g** was prepared from **8g** (90 mg, 0.25 mmol) according to the procedure described for **6a**. Recrystallization (MeOH/EtOAc) gave **6g** as a white solid (52 mg, 77%). mp 210–213 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.94 (1H, bs, OH), 9.70 (1H, bs, OH), 8.05–7.50 (6H, m, Ar-H and $CONH_2$), 7.36–7.05 (2H, m, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.73, 158.61, 157.91, 155.78, 129.56, 128.37, 128.12, 127.56, 127.11, 125.29, 121.64, 118.81, 111.69, 108.51. HRMS: m/z calcd for $C_{14}H_{11}N_2O_4$ $[M+H]^+$ 271.0719, found 271.0714.

Preparation of cRNAs of Housefly RDL and Their Expression in *Xenopus* Oocytes. The cRNAs of four housefly RDL variants (DDBJ accession Nos.: AB177547, complete cds of RDL_{bd}; AB824728, partial cds of exon 3a version; AB824729, partial cds of exon 6c version) were prepared as previously described.[20] In brief, the cDNA templates of four *Rdl* variants (ac, bc, ad, and bd) including the upstream RNA polymerase promoter site were

obtained by PCR amplification from pBluescript KS(-)-*MdRdl*$_{ac}$, -*MdRdl*$_{bc}$, -*MdRdl*$_{ad}$, and -*MdRdl*$_{bd}$ using KOD -Plus- Ver. 2 (Toyobo, Tokyo, Japan), a forward primer, 5'-TGTAAAACGACGGCCAGT-3', and a reverse primer 5'-CAGGAAACAGCTATGACC-3'. The PCR products were purified using the illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare UK, Ltd., Little Chalfont, UK), and the integrity of amplified cDNAs was confirmed by sequence analysis. The capped cRNAs were synthesized using T7 polymerase (mMessage mMachine® T7 Ultra Kit; Ambion, Austin, TX). The cRNAs were precipitated with LiCl, dissolved in sterile RNase-free water, and stored at -20 °C until use.

Mature female African clawed frogs (*Xenopus laevis*) were anesthetized with 0.1% (w/v) ethyl *m*-aminobenzoate methanesulfonate in water. The ovarian lobes were dissected out and then treated with collagenase (2 mg/mL, Sigma-Aldrich) in a Ca^{2+}-free standard oocyte solution (Ca^{2+}-free SOS) (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) at room temperature for 90–120 min. The oocytes were then gently washed with sterile SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) containing gentamycin (50 μg/mL, Life Technologies^{TM}, Thermo Fisher Scientific Inc., Waltham, MA), penicillin (100 U/mL, Life Technologies^{TM}), streptomycin (100 μg/mL, Life Technologies^{TM}), and sodium pyruvate (2.5 mM, Sigma-Aldrich). Each oocyte was injected cytoplasmically with 5 ng of cRNA dissolved in RNase-free water (9.2 nL), and then the oocytes were incubated in sterile SOS for 48 h at 16 °C.

Two-electrode Voltage Clamp (TEVC) Recordings. Electrophysiological experiments were performed as previously described.[14] Briefly, GABA-induced currents were recorded at

a holding potential of -80 mV using an Oocyte Clamp OC-725C amplifier (Warner Instruments, Hamden, CT) and Data-Trax2^{TM} software (World Precision Instruments Inc., Sarasota, FL). The glass capillary electrodes were fabricated using a pipette puller (P-97, Sutter Instrument, Novato, CA) and filled with 2 M KCl (resistance ranging from 0.5 to 2.0 MΩ). Oocytes were placed in a recording bath that was continuously perfused with SOS at 18–22 °C.

For agonist assays, GABA dissolved in SOS was applied to oocytes for 3 s, at intervals of 30–60 s to ensure a full recovery from desensitization. Concentration–response curves were generated by sequential applications of increasing concentrations of GABA. Muscimol hydrobromide was dissolved in SOS and tested using the same procedure as GABA. For antagonist assays, 3-isoxazolols were dissolved in DMSO and then diluted with SOS to the desired concentrations [DMSO, ≤ 0.1% (v/v)]. The test compound solution was added to the perfusate after two successive control applications of GABA and was then applied consecutively for the remainder of the experiments. Antagonist solutions were perfused alone for 30 s before their co-application with GABA. GABA, at a concentration corresponding to the EC_{50} for each variant, was then co-applied with an antagonist for 3 s, and the co-application was repeated at 30-60 s intervals to obtain the highest constant inhibition. All experiments were performed using at least four different oocytes obtained from at least two different frogs.

EC_{50} and IC_{50} values were obtained from concentration–response data by nonlinear regression analysis using OriginPro 8J (OriginLab, Northampton, MA). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by

294 Duncan's multiple range test.

295 **Insecticidal Assays.** The WHO/SRS strain of houseflies (*M. domestica*) was used to
296 examine the insecticidal activity of **6b**. Compound **6b** dissolved in DMSO (0.1 μL) at various
297 concentrations was injected into the dorsal side of the thorax of adult female houseflies (3–5
298 days after eclosion). This volume of DMSO solution alone did not affect the viability of
299 houseflies. Twelve to fifteen houseflies were used at each concentration. The injected
300 houseflies were maintained with sugar and water at 25 °C. The number of dead and/or
301 paralyzed flies was counted after 24 h. The experiments were replicated five times. The LD_{50}
302 value was calculated from the mean values of mortality at three dosages using the Probit
303 method.

304 **Molecular Modeling and Ligand Docking Studies.** A recently published X-ray crystal
305 structure of the homopentameric human β3 GABAR (PDB ID: 4COF)[21] was used as a
306 template to construct a housefly RDL_{ac} GABAR homology model. A sequence alignment of
307 the RDL_{ac} and β subunits was carried out using ClustalW software, and this was used to build
308 all five subunits simultaneously using MOE 2011.10 software (Chemical Computing Group,
309 Montreal, Canada). The obtained pentamer model was optimized geometrically using the
310 AMBER99 force field. GABA and muscimol were created in the zwitterionic forms, and
311 compound **6b** was created in a deprotonated hydroxyl form using MOE Builder. Potential
312 docking sites were searched using SiteFinder of MOE. The created ligands were docked into
313 the potential binding site of the generated model using the ASEDock program (2011.01.27,
314 Chemical Computing Group) with default parameters. The energies of the receptor and
315 ligands were minimized using the MMFF94x force field. The stable conformations of ligands

were obtained by a conformational search. The binding mode with the highest score was chosen for the final representation. Structural images were visualized using PyMOL Ver. 1.3 (Schrödinger, Tokyo, Japan).

RESULTS AND DISCUSSION

Chemistry. A new, convenient strategy to synthesize 4-aryl-substituted muscimols and 4-aryl-5-carbamoyl-3-isoxazolols starting from dimethyl acetylenedicarboxylate (**1**) was established and is outlined in Scheme 1. 3-Isoxazolol **2** was synthesized from **1** according to a reported method,[17] and the hydroxyl group was protected to give 3-benzyloxyisoxazole **3**.[18] Ammonolysis of ester **3** to amide **4** was followed by reduction with borane and deprotection with hydrobromic acid to afford muscimol hydrobromide (**5a**). Treatment of **4** with 30% hydrobromic acid in acetic acid afforded 5-carbamoyl-3-isoxazolol (**6a**). To introduce aromatic groups into the 4-position of the isoxazole ring, **4** was first iodinated at this position using iodine and $Pd(OAc)_2$ as a catalyst to give **7**.[22] The Suzuki-Miyaura cross-coupling reaction of **7** with the appropriate arylboronic acids in the presence of a palladium catalyst afforded analogs **8b**–**g** in 43–90% yields, the hydroxyl groups of which were deprotected with hydrobromic acid to give **6b**–**g**. Compounds **5b**–**c** were obtained by reduction and subsequent deprotection of **8b**–**c**, as described for **5a**.

Differential Sensitivity of Housefly GABAR Variants to GABA and Muscimol. Similar to the case of *D. melanogaster*,[5] four splice variants (ac, ad, bc, and bd) of RDL are endogenously generated by alternatively splicing exons 3 (a and b) and 6 (c and d) of *Rdl* in

houseflies. The two amino acid residues that differ between the sequences encoded by exons 3a and 3b are located upstream of the agonist-interacting region of the RDL subunit, and the ten residues that differ between the sequences encoded by exons 6c and 6d are located in a region including loops F and C (Figure S1), which are predicted to be generally involved in the interaction with agonists in Cys-loop receptors.[23] In the present study, the four variants of housefly RDL were expressed in *Xenopus* oocytes, and their responses to the agonists GABA and muscimol were first investigated using a TEVC method.

Application of GABA to the oocytes expressing housefly RDL GABARs induced concentration-dependent inward currents in all four variants when the voltage was clamped at –80 mV. The GABA concentration–response curves show that the order of variants giving higher sensitivity to GABA is $RDL_{ac} \approx RDL_{bc} > RDL_{ad} \approx RDL_{bd}$ (Figure 2A); the EC_{50} values are given in Table 1. The finding that the alternative splicing of exon 3 did not affect the sensitivity to GABA suggests that the two variable residues in exon 3 may not be involved in the interaction with GABA. RDL_{ac} GABARs showed ~6-fold higher sensitivity to GABA than RDL_{ad} GABARs, and RDL_{bc} GABARs had ~5-fold higher sensitivity than RDL_{bd} GABARs. This finding indicates that the alternative splicing of exon 6 significantly influences GABA potency in the RDL variants of housefly GABARs. Similarly, muscimol (**5a**) was a more potent full agonist in the ac and bc variants than in the ad and bd variants (Figure 2, Table 1). These different potencies of GABA and muscimol in the four variants are similar to those in *Drosophila* RDL variants.[6–8] The changes of agonist potencies may be due to the residue difference in loop(s) F and/or C in the orthosteric binding site.

Antagonism of Housefly GABAR Variants by Synthesized Analogs. We examined the

activity of synthesized compounds against RDL GABARs from three insect species. The compounds were first tested against common cutworm (*Spodoptera litura* (Fabricius)) and small brown planthopper (*Laodelphax striatellus* (Fallén)) RDL_{bd} GABARs expressed in *Drosophila* S2 cells using the fluorescent imaging plate reader (FLIPR®) membrane potential (FMP) assays. Unexpectedly, synthesized analogs other than muscimol failed to show significant activity at 100 μM in both insect receptors (data not shown). In contrast to these results, significant results were obtained against housefly RDL GABARs, prompting further investigation described below. These contrasting results suggest that structural or functional differences in the orthosteric binding sites of RDL GABARs might exist between insect species.

As agonist potencies vary by the variant, synthesized analogs were assessed for their functional characteristics in the four RDL variants of housefly GABARs expressed in *Xenopus* oocytes using a TEVC method. Analogs were first tested at 100 μM in the absence and in the presence of the EC_{50} of GABA to determine if they are agonists or antagonists. Unlike muscimol, all synthesized analogs showed no agonism but exhibited antagonism at 100 μM in the four RDL variants.

In a previous study, we found that the 4-(3-biphenylyl)-thio-4-PIOL and 4-(2-naphthyl)-thio-4-PIOL analogs showed competitive antagonism in housefly RDL_{ac} GABARs and common cutworm RDL_{bd} GABARs, indicating that the 3-biphenyl and 2-naphthyl groups are beneficial for competitive antagonists of insect GABARs.[14] Thus, these groups were introduced into the 4-position of muscimol (**5a**) to afford **5b** and **5c**. Both compounds showed antagonism at 100 μM in all four RDL variants of housefly GABARs,

albeit with less than 40% inhibition of GABA-induced currents (Figure 3A). Although the potencies of these analogs were low, these results indicate that the 3-isoxazolol scaffold may be useful for developing antagonists of housefly GABARs and that the bicyclic aromatic system at the 4-position of the isoxazole ring may be beneficial for antagonistic activity as it was in thio-4-PIOL analogs.

Replacement of the aminomethyl group of muscimol (**5a**) with a carbamoyl group to give **6a** changed the function of muscimol from an agonist to an antagonist in the four variants, with ~20% inhibition of GABA-induced currents at 100 μM in RDL_{ac} and RDL_{bc} GABARs and ~6% inhibition in RDL_{ad} and RDL_{bd} GABARs. These findings indicate that the protonated amino group is needed for agonist activity and that the carbamoyl group at the 5-position favors antagonism rather than agonism. The introduction of a 3-biphenylyl group at the 4-poisiton of **6a** to give **6b** markedly increased the antagonistic activity against the four variants, leading to 75.5, 76.1, 46.9, and 52.4% inhibition of GABA-induced currents in RDL_{ac}, RDL_{bc}, RDL_{ad}, and RDL_{bd} GABARs, respectively (Figure 3A). Figure 3B shows that GABA-induced currents were inhibited by the 3-biphenylyl analog (**6b**) at 100 μM in the four RDL variants. Compound **6c**, with a 2-naphthyl substitution at the 4-position of the isoxazole ring, also exhibited higher inhibition than **6a** in all four variants, but relatively lower inhibition compared with **6b**. Additionally, two different aromatic groups, 4-biphenylyl and 1-naphthyl, were introduced into the 4-position to yield **6d** and **6e**, respectively. The inhibition of GABA-induced currents by **6d** and **6e** in the four variants was comparable to the inhibition by **6b**. These findings indicate that analogs with bicyclic aromatic groups at the 4-position of the isoxazole ring are well tolerated at the binding site and that they are

404 effective in inhibiting GABA-induced current in housefly GABARs. To investigate whether
405 the electron-donating groups on the aromatic group at the 4-position increase activity, a
406 methoxyl and a hydroxyl group were introduced to the 6-position of the 2-naphthyl group of
407 **6c** to afford **6f** and **6g**, respectively. Compounds **6f** and **6g** were comparable in activity to **6c**
408 in the four variants, although inhibition by **6f** in the ac variant was higher than that of **6c**
409 (Figure 3A). Overall, the synthesized analogs **5b**–**c** and **6b**–**g** were more potent against
410 RDL_{ac} and RDL_{bc} GABARs than RDL_{ad} and RDL_{bd} GABARs (Figure 3A), with ~1.5- to
411 ~4-fold higher inhibition in the former than in the latter. The different sensitivities of the four
412 variants to the synthesized analogs are similar to those of the agonists GABA and muscimol,
413 implying that the synthesized analogs bind to the same site as agonists.

414 Compounds **6b**–**g**, which exerted relatively greater inhibitory effects, were further
415 evaluated in RDL_{ac} GABARs with the generation of antagonist concentration–response
416 curves in the presence of 10 μM (EC_{50}) GABA (Figure 4A). The 3-biphenylyl analog (**6b**),
417 with an IC_{50} value of 30.0 μM, is among the analogs that displayed the greatest antagonism in
418 the ac variant (Table 2). The replacement of the 3-biphenylyl group with a 2-naphthyl and a
419 4-biphenylyl group to yield **6c** and **6d** resulted in 2.3- and 1.8-fold increases in the IC_{50} value,
420 respectively. The 1-naphthyl analog (**6e**) had a potency similar to that of **6b** in the ac variant.
421 Thus, the 3-biphenyl group is advantageous compared with the 4-biphenyl group; the
422 1-naphthyl group is preferable to the 2-naphthyl group. The introduction of a methoxy group
423 into the naphthyl group of **6c** to yield **6f** led to a 1.9-fold increased potency in the ac variant,
424 whereas the potency of **6g**, in which a hydroxyl group was introduced, was similar to that of
425 **6c** in the ac variant.

Compound **6b** was further examined for its potencies in other RDL variants of housefly GABARs. Figure 4B shows concentration–response curves for **6b** in the presence of the EC_{50} of GABA in the four variants. No significant differences were observed in the IC_{50} values of **6b** between the ac and bc variants or between the ad and bd variants, whereas the IC_{50} values of **6b** in the ad and bd variants were ~3-fold greater compared with those in the ac and bc variants (Table 2). These findings were analogous to those in the EC_{50} values of GABA and muscimol in the four variants, indicating that **6b** most likely acts on the same site as agonists. The different amino acid residues in the region encoded by exon 6 in different variants may cause the difference in the sensitivity to competitive antagonists and agonists.

Mode of Antagonism. To determine whether synthesized 3-isoxazolols act as competitive antagonists, the GABA concentration–response relationships in the presence and absence of **6b** were examined in the ac variant. The GABA concentration–response curves made a parallel rightward shift with increasing **6b** concentrations, indicating a competitive mechanism (Figure 5). The EC_{50} values of GABA in the absence and presence of 30 and 100 μM **6b** were 10.6, 27.2, and 55.6 μM, respectively. The potency of GABA was decreased 2.6- and 5.2-fold in the presence of 30 and 100 μM **6b**, respectively, whereas the efficacy of GABA remained unchanged. These results indicate that **6b** competes with GABA for the orthosteric site to stabilize the closed conformation of chloride channels.

Insecticidal Activity. Competitive antagonists stabilize the closed conformation of GABAR channels and should thus exert insecticidal effects when they act at insect GABARs. There is no information about the insecticidal action of competitive antagonists. We therefore investigated whether **6b**, which showed the highest antagonism, has intrinsic insecticidal

activity by injection into adult female houseflies. As a result, **6b** proved to be insecticidal, with an LD_{50} value of 5.6 (4.9–6.3) nmol/fly (95% confidence interval in parenthesis). The finding that **6b** shows insecticidal activity by definition is somewhat encouraging, although the activity was not prominent and was observed by injection but not topical application.

Molecular Interaction between Ligands and Housefly RDL GABAR. To understand the molecular mechanisms of the interaction between 3-isoxazolols and insect GABARs, GABA, muscimol, and the 3-biphenylyl analog (**6b**) were docked into the orthosteric binding site of a housefly RDL_{ac} GABAR homology model constructed based on the X-ray crystal structure of the human homopentameric β3 GABAR. The β3 subunit shares 38.7% amino acid identity with the RDL_{ac} subunit. The most likely binding poses were selected based on the score and shown in Figure 6. The orthosteric site of Cys-loop receptors, which is located in the extracellular interface of two adjacent subunits (Figure S2), is formed by loops A–C from the principal subunit and loops D–F from the complementary subunit.[24] Similarly, the orthosteric binding pocket of the constructed model is basically formed by Phe144, Val146, Glu202, Ser203, Phe204, Gly205, Ile245, Leu247, and Arg254 from the principal subunit and by Tyr88, Leu90, Tyr107, Arg109, and Met224 from the complementary subunit. The distance between two key residues, Glu202 and Arg109, is approximately 9.0 Å.

The docking simulation showed that the protonated amino groups of GABA and muscimol are located close to Glu202 of loop B and that they form an electrostatic interaction and hydrogen bonds with this residue (Figures 6A, B). Arg109 of loop D electrostatically interacts with the deprotonated carboxyl group of GABA and the deprotonated hydroxyl group (or the isoxazole ring) of muscimol. In addition, Arg109 serves as a hydrogen bond

donor for the carboxylate of GABA and the hydroxyl oxygen or the nitrogen atom of muscimol. These important interactions were observed in the docking simulation results using another housefly RDL GABAR homology model in our previous studies and are apparently conserved in *Drosophila* RDL GABARs.[14,15,25–27] Tyr252 of loop C, which surrounds the protonated amino group of GABA and muscimol, may produce a cation-π interaction, as proposed for *Drosophila* RDL GABARs.[25–27] Similar orientations and interactions of GABA and muscimol in the binding site indicate that these two agonists interact with housefly RDL GABARs in an identical mode.

Docking of the 4-(3-biphenylyl) analog (**6b**) to the homology model of the housefly RDL_{ac} GABAR predicts that the 5-carbamoyl-3-isoxazolol scaffold of **6b** lies between Glu202 (loop B) and Arg109 (loop D) in the same orientation as muscimol. Similar to the cases of GABA and muscimol, the side chain of Arg109 electrostatically interacts with the deprotonated hydroxyl group of **6b** and forms a hydrogen bond with the same group; the side chain of Glu202 functions as a hydrogen acceptor for the carbamoyl group of **6b**. Furthermore, the two amino acid residues, Arg254 of loop C and Tyr107 of loop D, surround the 3-biphenylyl group of **6b**. The present docking studies of **6b** predict that the 3-biphenylyl group points out of the binding site and may form a π-cation interaction with Arg254 and a π-π interaction with Tyr107. The orientation of the 3-biphenylyl group is in contrast with that of the 4-substitution of thio-4-PIOL analogs in our previous study.[14] This difference may be due to the different templates used in the homology modeling. It has yet to be elucidated which orientation is feasible.

In conclusion, we synthesized a novel class of housefly competitive GABAR antagonists (**6b**–**g**) by replacing the aminomethyl group of muscimol with a carbamoyl group and simultaneously introducing bicyclic aromatic groups at the 4-position. All of the analogs exhibited antagonism of the four splice variants of housefly GABARs, the most potent compound being 4-(3-biphenylyl)-5-carbamoyl-3-isoxazolol (**6b**). The potencies of **6b** in RDL_{ac} and RDL_{bc} GABARs were ~3-fold greater than those in RDL_{ad} and RDL_{bd} GABARs, and this potency difference in these variants is similar to the potency difference of agonists. The identification of a novel series of competitive GABAR antagonists serves to widen the current scope for insecticidal chemicals competitively acting at the orthosteric sites beyond those of gabazine and thio-4-PIOL derivatives.

ASSOCIATED CONTENT

Supporting Information

Alignments of amino acid sequences encoded by exons 3a, 3b, 6c, and 6d of housefly *Rdl* (Figure S1). View of the orthosteric binding pocket in a housefly RDL_{ac} GABAR homology model (Figure S2). The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org/.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ozoe-y@life.shimane-u.ac.jp. Phone: +81-852-32-6573. Fax: +81-852-32-6573.

Funding

This work was supported in part by JSPS KAKENHI Grant Number 26292031.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. I. Ikeda, and Mr. K. Nomura (Faculty of Life and Environmental Science, Shimane University) for their useful advice and technical assistance.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; EC_{50}, half maximal effective concentration; GABA, γ-aminobutyric acid; GABAR, GABA receptor; IC_{50}, half maximal inhibitory concentration;

523 LD_{50}, median lethal dose; PCR, polymerase chain reaction; RDL, name for an insect GABAR
524 subunit; SOS, standard oocyte solution; TEVC, two-electrode voltage clamp; Thio-4-PIOL,
525 5-(4-piperidyl)-3-isothiazolol

REFERENCES

(1) Purves, D.; Augustine, G. J.; Fitzpatrick, D.; Hall, W. C.; LaMantia, A.-S.; White, L. E. In *Neuroscience*, Fifth edition; Sinauer Associates: Sunderland, MA, 2012; pp 25–140.

(2) Ozoe, Y. γ-Aminobutyrate- and glutamate-gated chloride channels as targets of insecticides. In *Advances in Insect Physiology*; Ephraim, C., Ed.; Academic Press: Burlington, MA, 2013; Vol. 44, pp 211–286.

(3) Ozoe, Y.; Takeda, M.; Matsuda, K. γ-Aminobutyric acid receptors: a rationale for developing selective insect pest control chemicals. In *Biorational Control of Arthropod Pests*; Ishaaya, I., Horowitz, A. R., Eds.; Springer: Heidelberg, DE, 2009; pp 131–162.

(4) Buckingham, S.; Sattelle, D. GABA receptors of insects. In *Insect Pharmacology: Channels, Receptors, Toxins and Enzymes*; Gilbert, L. I., Gill, S. S., Eds.; Academic Press: London, UK, 2010; pp 29–64.

(5) ffrench-Constant, R. H.; Rocheleau, T. A. *Drosophila* γ-aminobutyric acid receptor gene *Rdl* shows extensive alterntive splicing. *J. Neurochem.* **1993**, *60*, 2323–2326.

(6) Hosie, A. M.; Sattelle, D. B. Agonist pharmacology of two *Drosophila* GABA receptor splice variants. *Br. J. Pharmacol.* **1996**, *119*, 1577–1585.

(7) Hosie, A. M.; Buckingham, S. D.; Presnail, J. K.; Sattelle, D. B. Alternative splicing of a *Drosophila* GABA receptor subunit gene identifies determinants of agonist potency. *Neuroscience* **2001**, *102*, 709–714.

(8) Jones, A. K.; Buckingham, S. D.; Papadaki, M.; Yokota, M.; Sattelle, B. M.; Matsuda, K.; Sattelle, D. B. Splice-variant- and stage-specific RNA editing of the *Drosophila*

548 GABA receptor modulates agonist potency. *J. Neurosci.* **2009**, *29*, 4287–4292.
549 (9) Ozoe, Y.; Asahi, M.; Ozoe, F.; Nakahira, K.; Mita, T. The antiparasitic isoxazoline
550 A1443 is a potent blocker of insect ligand-gated chloride channels. *Biochem. Biophys.*
551 *Res. Commun.* **2010**, *391*, 744–749.
552 (10) Lahm, G. P.; Cordova, D.; Barry, J. D.; Pahutski, T. F.; Smith, B. K.; Long, J. K.;
553 Benner, E. A.; Holyoke, C. W.; Joraski, K.; Xu, M.; Schroeder, M. E.; Wagerle, T.;
554 Mahaffey, M. J.; Smith R. M.; Tong. M.-H. 4-Azolylphenyl isoxazoline insecticides
555 acting at the GABA gated chloride channel. *Bioorg. Med. Chem. Lett.* **2013**, *23*,
556 3001–3006.
557 (11) Nakao, T.; Banba, S.; Nomura, M.; Hirase, K. Meta-diamide insecticides acting on
558 distinct sites of RDL GABA receptor from those for conventional noncompetitive
559 antagonists. *Insect Biochem. Mol. Biol.* **2013**, *43*, 366–375.
560 (12) Ozoe, Y.; Kita, T.; Ozoe, F.; Nakao, T.; Sato, K.; Hirase, K. Insecticidal
561 3-benzamido-*N*-phenylbenzamides specifically bind with high affinity to a novel
562 allosteric site in housefly GABA receptors. *Pestic. Biochem. Physiol.* **2013**, *107*,
563 285–292.
564 (13) Casida, J. E. Golden age of RyR and GABA-R diamide and isoxazoline insecticides:
565 common genesis, serendipity, surprises, selectivity, and safety. *Chem. Res. Toxicol.*
566 **2015**, *28*, 560–566.
567 (14) Liu, G.; Furuta, K.; Nakajima, H.; Ozoe, F.; Ozoe, Y. Competitive antagonism of insect
568 GABA receptors by 4-substituted 5-(4-piperidyl)-3-isothiazolols. *Bioorg. Med. Chem.*
569 **2014**, *22*, 4637–4645.

570 (15) Rahman, M. M.; Liu, G.; Furuta, K.; Ozoe, F.; Ozoe, Y. Synthesis of 1,3-di- and
571 1,3,4-trisubstituted 1,6-dihydro-6-iminopyridazines as competitive antagonists of insect
572 GABA receptors. *J. Pestic. Sci.* **2014**, *39*, 133–143.
573 (16) Rahman, M. M.; Akiyoshi, Y.; Furutani, S.; Matsuda, K.; Furuta, K.; Ikeda, I.; Ozoe, Y.
574 Competitive antagonism of insect GABA receptors by iminopyridazine derivatives of
575 GABA. *Bioorg. Med. Chem.* **2012**, *20*, 5957–5964.
576 (17) Frey, M.; Jäger, V. Synthesis of *N*-substituted muscimol derivatives including
577 *N*-glycylmuscimol. *Synthesis* **1985**, *12*, 1100–1104.
578 (18) Riess, R.; Schön, M.; Laschat, S.; Jäger, V. Evaluation of protecting groups for
579 3-hydroxyisoxazoles – short access to 3-alkoxyisoxazole-5-carbaldehydes and
580 3-hydroxyisoxazole-5-carbaldehyde, the putative toxic metabolite of muscimol. *Eur. J.*
581 *Org. Chem.* **1998**, *3*, 473–479.
582 (19) Krogsgaard-Larsen, P.; Christensen, S. B. Structural analogues of GABA. A new
583 convenient synthesis of muscimol. *Acta Chem. Scand. B* **1976**, *30*, 281–282.
584 (20) Eguchi, Y.; Ihara, M.; Ochi, E.; Shibata, Y.; Matsuda, K.; Fushiki, S.; Sugama, H.;
585 Hamasaki, Y.; Niwa, H.; Wada, M.; Ozoe, F.; Ozoe, Y. Functional characterization of
586 *Musca* glutamate- and GABA-gated chloride channels expressed independently and
587 coexpressed in *Xenopus* oocytes. *Insect Mol. Biol.* **2006**, *15*, 773–783.
588 (21) Miller, P. S.; Aricescu, A. R. Crystal structure of a human $GABA_A$ receptor. *Nature*
589 **2014**, *512*, 270–275.
590 (22) Wang, X. C.; Hu, Y.; Bonacorsi, S.; Hong, Y.; Burrell, R.; Yu, J. Q. Pd(II)-catalyzed
591 C–H iodination using molecular I_2 as the sole oxidant. *J. Am. Chem. Soc.* **2013**, *135*,

592 10326–10329.
593 (23) Buckingham, S. D.; Biggin, P. C.; Sattelle, B. M.; Brown, L. A.; Sattelle, D. B. Insect
594 GABA receptors: splicing, editing, and targeting by antiparasitics and insecticides. *Mol.*
595 *Pharmacol.* **2005**, *68*, 942–951.
596 (24) Miller, P. S.; Smart, T. G. Binding, activation and modulation of Cys-loop receptors.
597 *Trends Pharmacol. Sci.* **2010**, *31*, 161–174.
598 (25) Ashby, J. A.; McGonigle, I. V.; Price, K. L.; Cohen, N.; Comitani, F.; Dougherty, D. A.;
599 Molteni, C.; Lummis, S. C. R. GABA binding to an insect GABA receptor: a molecular
600 dynamics and mutagenesis study. *Biophys. J.* **2012**, *103*, 2071–2081.
601 (26) Comitani, F.; Cohen, N.; Ashby, J.; Botten, D.; Lummis, S. C. R.; Molteni, C. Insights
602 into the binding of GABA to the insect RDL receptor from atomistic simulations: a
603 comparison of models. *J. Comput. Aided Mol. Des.* **2014**, *28*, 35–48.
604 (27) McGonigle, I.; Lummis, S. C. R. Molecular characterization of agonists that bind to an
605 insect GABA receptor. *Biochemistry* **2010**, *49*, 2897–2902.

606 **Figure captions**

607

608 **Figure 1.** Chemical structures of GABA, muscimol, thio-4-PIOL, gabazine, and the
609 synthesized analogs. Bold blue lines represent the GABA structural scaffold.

610

611 **Figure 2.** Agonist responses of the four splice variants of housefly RDL GABARs expressed
612 in *Xenopus* oocytes. Responses were normalized relative to the maximum currents induced
613 by 1 mM GABA in the ac and bc variants and by 3 mM GABA in the ad and bd variants. No
614 significant differences were observed in the maximum currents induced by muscimol and
615 GABA in each variant, indicating that muscimol is a full agonist. Data represent means ±
616 SEM (n = 6–9). (A) GABA concentration–response curves of the four variants. (B) Muscimol
617 concentration–response curves of the four variants.

618

619 **Figure 3.** Inhibition of GABA-induced currents by the 3-isoxazolol analogs in the four splice
620 variants of housefly RDL GABARs expressed in *Xenopus* oocytes. The EC_{50} of GABA for
621 each variant (Table 1) was used to induce control currents in each oocyte. (A) Inhibition of
622 GABA-induced currents by synthesized analogs at 100 μM in the four variants. Data
623 represent means ± SEM (n = 4–6). (B) Examples of GABA-induced currents inhibited by 100
624 μM **6b** in the four variants.

625

626 **Figure 4.** Effects of the 3-isoxazolol analogs on GABA-induced currents in housefly RDL
627 GABARs expressed in *Xenopus* oocytes. Data represent means ± SEM (n = 4–6). (A)

628 Concentration–response inhibition curves of 4-aryl-5-carbamoyl-3-isoxazolols (**6b**–**g**) in the
629 housefly RDL_{ac} variant. The EC_{50} (10 μM) of GABA was used to induce the currents. (B)
630 Concentration–response inhibition curves of **6b** in four GABAR variants. Responses were
631 normalized relative to currents induced by the EC_{50} of GABA for each variant.
632
633 **Figure 5.** GABA concentration–response curves of housefly RDL_{ac} GABARs in the presence
634 and absence of 30 and 100 μM **6b**. Responses were normalized relative to the maximum
635 current induced by 1 mM GABA in each oocyte. Data represent means ± SEM (n = 4–6).
636
637 **Figure 6.** Simulation of the docking of GABA, muscimol, and **6b** into the orthosteric binding
638 site of a housefly RDL_{ac} GABAR homology model. The crystal structure of the
639 homopentameric human β3 GABAR (PDB: 4COF) was used as a template to construct the
640 model. (A) Docking of GABA into the orthosteric site. (B) Docking of muscimol. (C)
641 Docking of **6b**.
642
643 **Scheme 1.** Synthesis of muscimol and target compounds.[a]
644 [a]Reagents and conditions: (a) *N*-hydroxyurea, 1,5-diazabicyclo[5.4.0]undec-5-ene, MeOH,
645 0 °C; (b) benzyl bromide, K_2CO_3, acetone, 70 °C; (c) aqueous NH_3, room temperature; (d)
646 BH_3, THF, room temperature; (e) 30% HBr in AcOH, room temperature; (f) I_2, $Pd(OAc)_2$,
647 CsOAc, $NaHCO_3$, DMF, 75 °C; (g) $RB(OH)_2$, $PdCl_2(PPh_3)_2$, K_2CO_3, DMF, 80 °C.

Table 1. Potencies of GABA and Muscimol in the Four Splice Variants of Housefly RDL GABARs

Variant	GABA		Muscimol	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
RDL_{ac}	10.6 ± 1.6[a]	1.74 ± 0.23	7.1 ± 0.7[a]	1.25 ± 0.16
RDL_{bc}	12.2 ± 0.7[a]	1.62 ± 0.24	10.2 ± 1.0[a]	1.18 ± 0.10
RDL_{ad}	64.1 ± 3.8[b]	2.29 ± 0.10	54.1 ± 2.2[b]	1.98 ± 0.26
RDL_{bd}	59.0 ± 5.7[b]	1.77 ± 0.21	45.8 ± 4.7[b]	1.76 ± 0.16

Data are means ± SEM (n = 6–9). n_H is the Hill coefficient. The different superscript letters within a column indicate statistically significant difference with $p < 0.01$.

653 **Table 2. Inhibition of GABA (EC_{50})-induced Currents by**
654 **4-Aryl-5-carbamoyl-3-isoxazolols (6b–g) in the Four Variants of Housefly RDL**
655 **GABARs**

Comp.	IC_{50} (μM)			
	RDL_{ac}	RDL_{bc}	RDL_{ad}	RDL_{bd}
6b	30.0 ± 2.6^{a}	34.3 ± 2.4^{a}	107.2 ± 8.1^{b}	96.0 ± 4.9^{b}
6c	67.7 ± 3.0^{c}	> 100	> 100	> 100
6d	53.3 ± 3.4^{d}	ND	> 100	> 100
6e	38.5 ± 4.9^{a}	ND	≈ 100	> 100
6f	36.0 ± 3.5^{a}	ND	> 100	> 100
6g	64.9 ± 2.7^{cd}	> 100	> 100	> 100

656 Data are means ± SEM (n = 4). The different superscript letters within a row and a
657 column indicate statistically significant difference with $p < 0.01$ and $p < 0.05$,
658 respectively. ND: not determined.

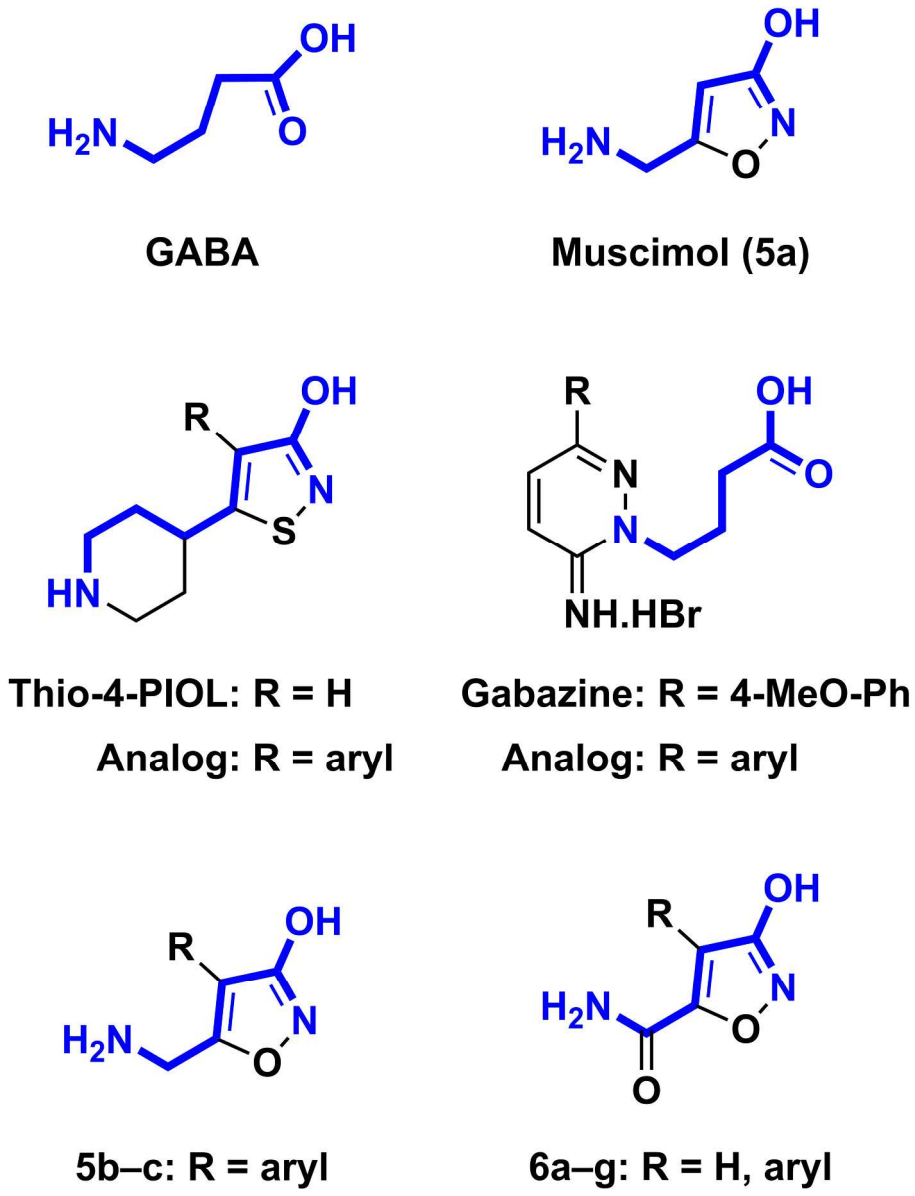


Figure 1. Chemical structures of GABA, muscimol, thio-4-PIOL, gabazine, and the synthesized analogs.
108x141mm (600 x 600 DPI)

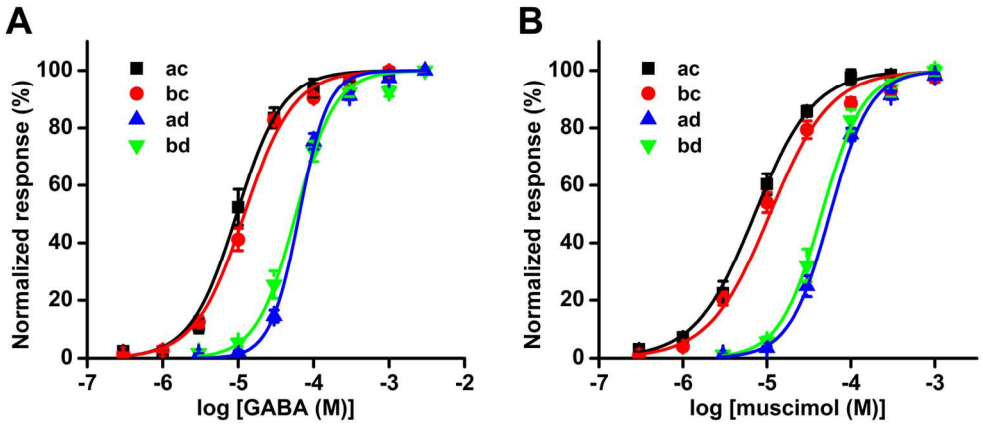


Figure 2. Agonist responses of the four splice variants of housefly RDL GABARs expressed in *Xenopus* oocytes.
76x33mm (600 x 600 DPI)

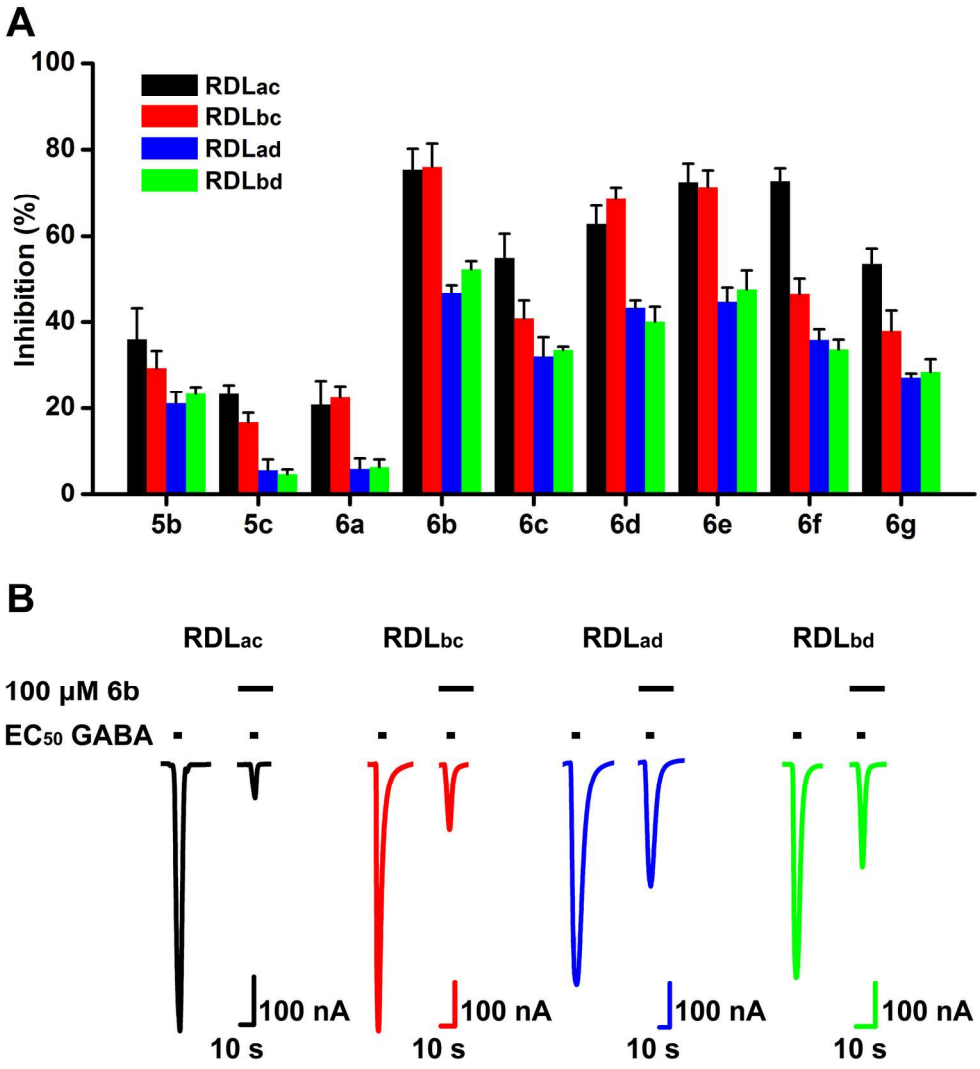


Figure 3. Inhibition of GABA-induced currents by the 3-isoxazolol analogs in the four splice variants of housefly RDL GABARs expressed in *Xenopus* oocytes.
169x182mm (300 x 300 DPI)

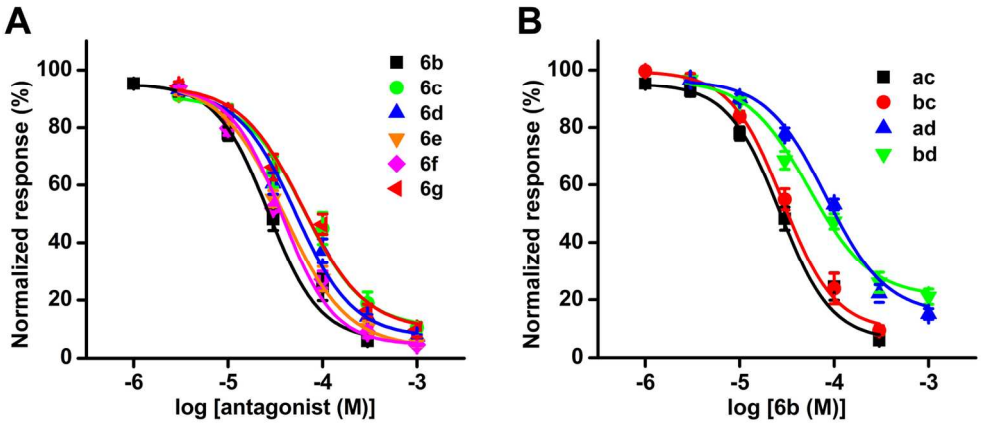


Figure 4. Effects of the 3-isoxazolol analogs on GABA-induced currents in housefly RDL GABARs expressed in *Xenopus* oocytes.
76x33mm (600 x 600 DPI)

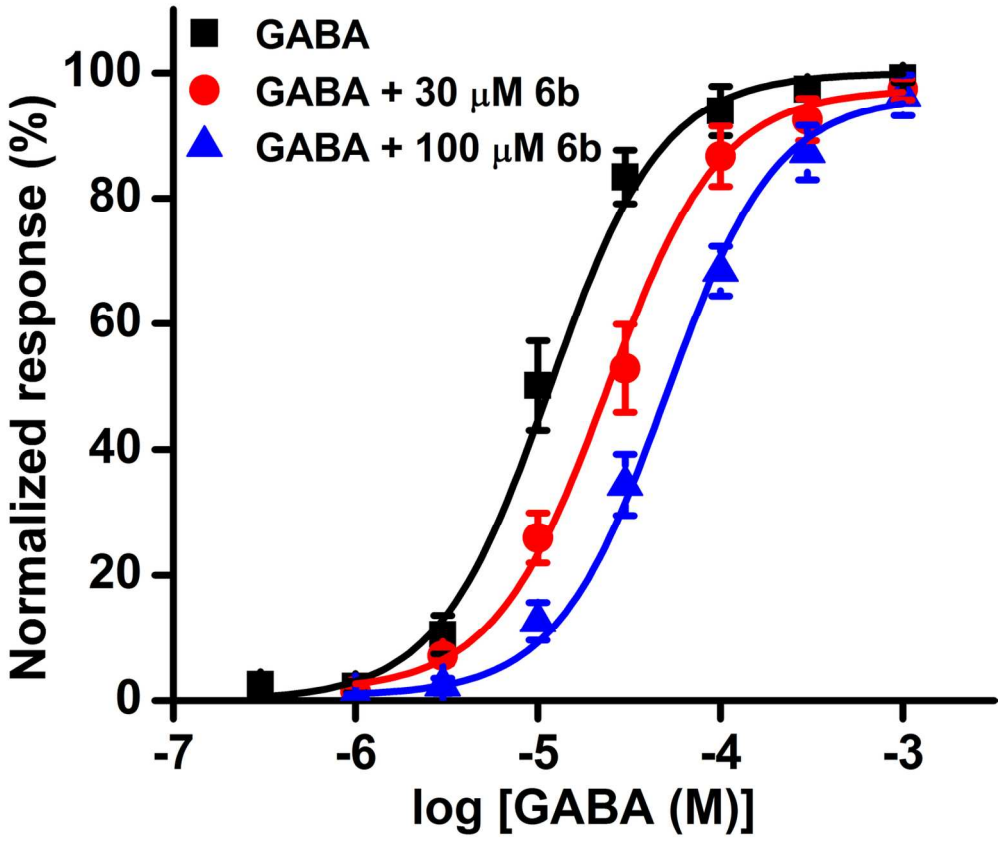


Figure 5. GABA concentration–response curves of housefly RDL_{ac} GABARs in the presence and absence of 30 and 100 μM of **6b**.
67x56mm (600 x 600 DPI)

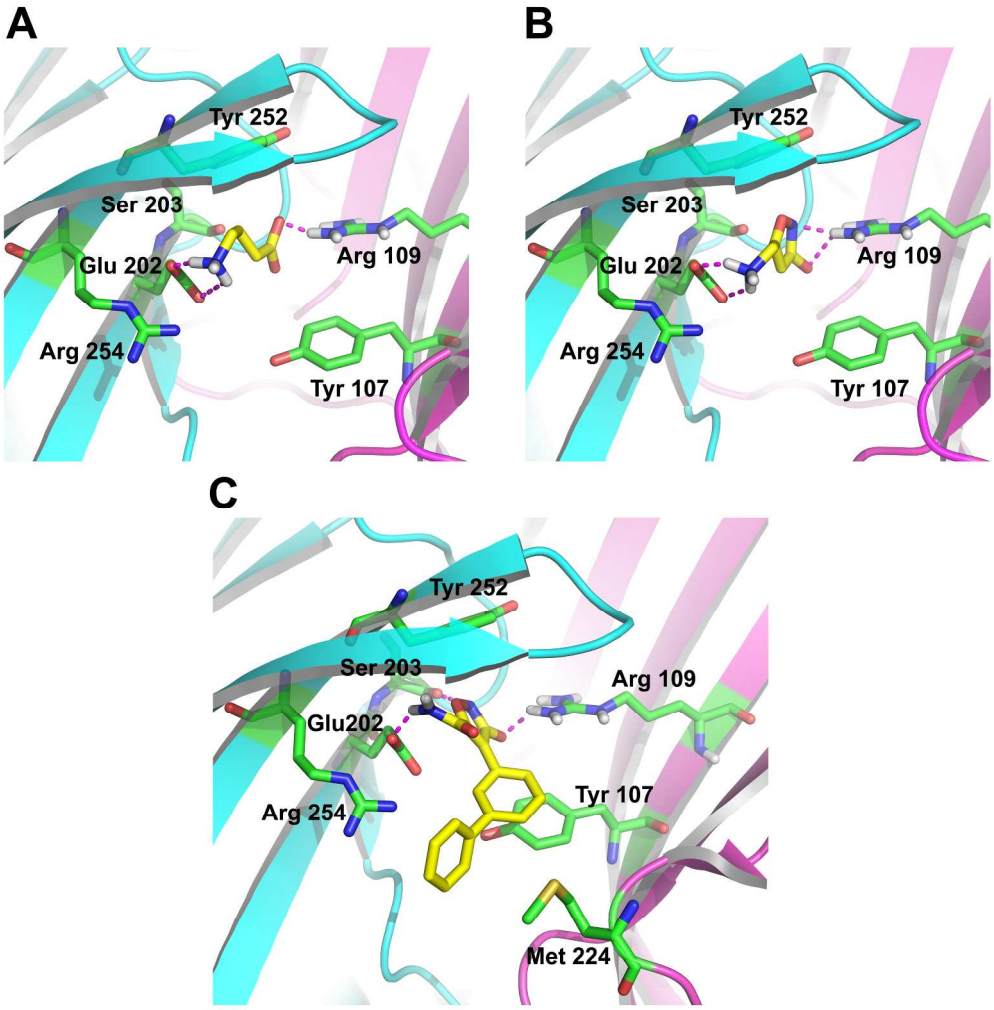
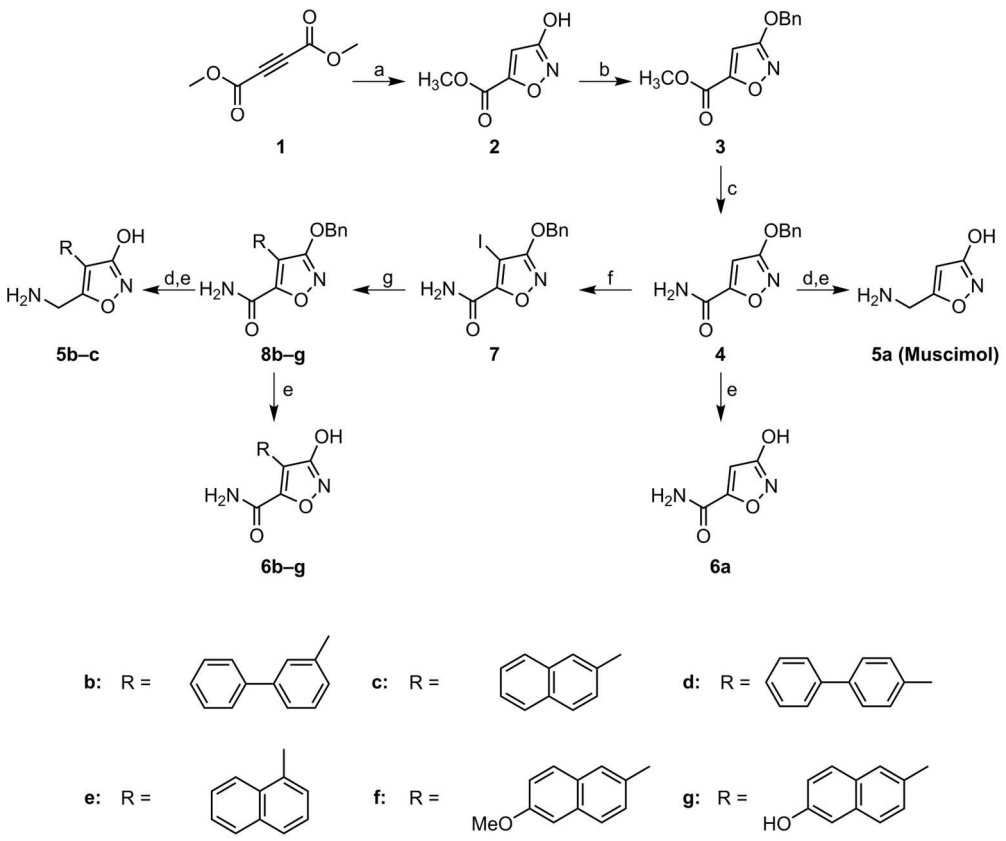
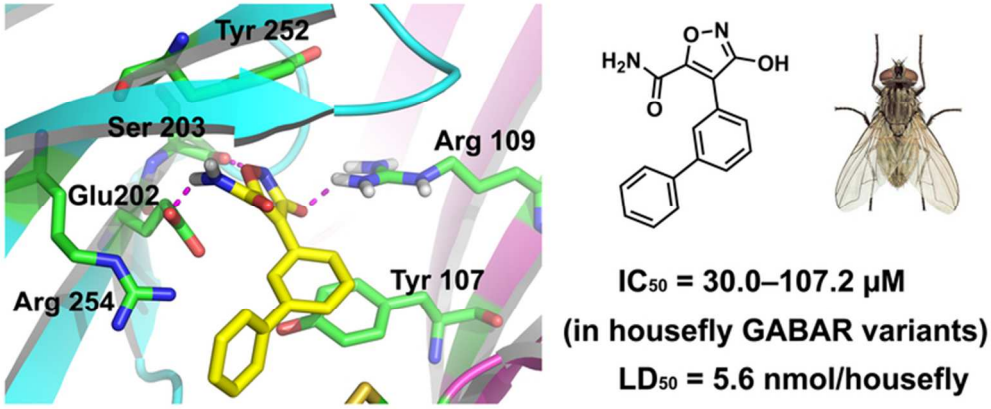


Figure 6. Simulation of the docking of GABA, muscimol, and **6b** into the orthosteric binding site of a housefly RDL_{ac} GABAR homology model.
250x255mm (300 x 300 DPI)



Scheme 1. Synthesis of muscimol and target compounds.
153x128mm (300 x 300 DPI)



Graphic for Table of Contents
33x14mm (600 x 600 DPI)