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Receptor Assay-Guided Isolation of Anti-GABAergic Insecticidal Alkaloids from a Fungal Culture

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The γ-aminobutyric acid (GABA) receptor bears sites of action for insecticides. To discover GABA receptor-directed insecticides in natural products, fungal culture extracts were screened for their ability to inhibit specific binding of the radiolabeled noncompetitive antagonist [^{3}H]1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane to housefly head membranes. The screening efforts led to the isolation of two alkaloids from *Aspergillus terreus*: PF1198A (alantrypinone) and PF1198B (serantrypinone), which had IC_{50} values of 0.34 and 2.1 μM, respectively, in this assay. These compounds were ca. 47−61-fold selective for housefly vs rat GABA receptors. Both compounds showed insecticidal activity against *Myzus persicae* in the range of 100−500 ppm. Binding assay-guided screening should provide significant opportunities for the identification of novel and selective insecticides.

KEYWORDS: GABA receptor; insecticidal compounds; PF1198; alantrypinone; serantrypinone

INTRODUCTION

The ionotropic GABA receptor mediates fast neurotransmission in inhibitory synapses of the nervous system of animals. This receptor plays a pivotal role in the regulation of the excitability of the nervous system. There are multiple binding sites for modulators, as well as for agonists and antagonists, on the GABA receptor (*1*). Ghiasuddin and Matsumura were the first to suggest the implications of the GABA receptor in the insecticidal action of lindane and cyclodienes (*2*). It has been established by subsequent extensive studies that the chlorinated insecticides act as noncompetitive GABA receptor antagonists (*3*). Later, the phenylpyrazole insecticide fipronil was also found to selectively act on insect GABA receptors in a fashion similar to that of the chlorinated insecticides, although it remains to be determined whether their binding sites are identical (*4*). In addition, we previously reported that naturally occurring terpenoids exhibited insecticidal activity by binding to the antagonist site of the GABA receptor (*5*−*8*). Several of the terpenoids were revealed to be selective for insect GABA receptors over mammalian receptors. Furthermore, GABA receptors are also a target for macrolide insecticides such as avermectins (*9*). These findings prompted us to screen for highly selective, insecticidal GABA receptor ligands from fungal cultures. To this end, we employed [^{3}H]EBOB, an antagonist ligand for GABA receptors (*10*), as a probe. We report here the isolation of two alkaloids fitting the definition of such compounds.

MATERIALS AND METHODS

Chemicals. [^{3}H]EBOB was purchased from NEN Life Science Products, Inc. (1.11 TBq/mmol). EBOB was synthesized according to the method of Palmer and Casida (*11*). Wakogel C-300 was obtained from Wako Pure Chemical Industries, Ltd. Silica gel 60 F_{254} was obtained from Merck, Ltd.

Isolation of [^{3}H]EBOB Binding Inhibitors. A 65% acetone solution in water (16 L) was added to strain PF1198 cultured in a rice medium (8 kg). The mixture was filtered, and the acetone of the filtrate (15 L) was evaporated. The remaining aqueous solution (8 L) was extracted with ethyl acetate (10 L). The extract was concentrated and subjected to silica gel column chromatography (Wakogel C-300, 1 kg; eluent, chloroform/methanol 20:1). A solid mass (950 mg) from fractions that produced inhibition in [^{3}H]EBOB binding assays using housefly head membranes was recrystallized from diethyl ether/methanol to give a mixture of inhibitors (700 mg). Two inhibitors with R_f values of 0.57 (PF1198A, 111 mg) and 0.46 (PF1198B, 56 mg) were isolated from the mixture by preparative thin-layer chromatography (silica gel 60 F_{254}) with *n*-hexane/acetone (1:1).

[^{3}H]EBOB Binding Assays Using Housefly Head and Rat Brain Membranes. For the preparation of housefly head P_2 membranes, the heads of adult houseflies (*Musca domestica* L.) were homogenized in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose (buffer A) with a Teflon glass homogenizer, and the homogenate was centrifuged at 500*g* for 5 min after filtration through four layers of 64 μm mesh screen. The supernatant was centrifuged at 25 000*g* for 30 min after filtration through the screen. The resulting pellets were resuspended in buffer A and were kept on ice for 30 min. The

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suspension was recentrifuged at 25 000*g* for 30 min, and the resulting pellets were finally suspended in 10 mM phosphate buffer (pH 7.5) containing 300 mM NaCl (buffer B) and were used immediately thereafter for the binding assays.

For the preparation of rat brain P_2 membranes, whole brains of 5 week old male Wistar rats were homogenized in 1 mM ethylenediaminetetraacetic acid (EDTA) using a Teflon glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was then centrifuged at 25 000*g* for 30 min. The resulting pellets were suspended in 1 mM EDTA, packed into cellophane tubing, and dialyzed against distilled/deionized water in an ice bath (1−2 L, 2 h × 3). The inner suspension was then centrifuged at 25 000*g* for 30 min, and the pellets were stored at −80 °C. On the day of the binding experiments, the pellets were suspended in buffer B.

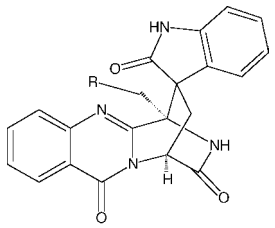
The [^{3}H]EBOB binding assays were carried out as follows. Fungal culture extracts dissolved in methanol (2 μL) or purified compounds dissolved in dimethyl sulfoxide (DMSO, 4 μL) were incubated with housefly head membranes (200 μg of protein) or rat brain membranes (125 μg of protein) and 0.5 nM [^{3}H]EBOB in 1.0 mL of buffer B at 22 °C for 70 min (housefly) or at 37 °C for 90 min (rat). After they were incubated, the mixtures were filtered through GF/B filters and rapidly rinsed twice with 5 mL of buffer B using a Brandel M-24 cell harvester. The radioactivity of [^{3}H]EBOB that specifically had bound to membranes on the filters was measured with a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5 μM unlabeled EBOB. Each experiment was performed in triplicate and repeated three times.

Insecticidal Assays. In preliminary assays using the housefly (*M. domestica* L.), a 2:1 mixture of PF1198A and -B was topically applied with acetone (1 μL) on the dorsal surface of the thorax of 15 female adults 1 h after the pretreatment of a 1 μL acetone solution containing 10 μg of piperonyl butoxide. In assays using the German cockroach (*Blattella germanica* L.), the same PF1198 mixture was injected with DMSO (0.25 μL) into the ventral portion of the abdomen of male adults (10−20 roaches for each dose) pretreated with piperonyl butoxide (30 μg). The mortality determinations were made 24 h after the treatments in assays using houseflies and cockroaches. For assays using the green peach aphid (*Myzus persicae* Sulzer), a cabbage leaf disk (28 mm in diameter) was placed on an agar plate in a plastic dish (50 mm in diameter). Each test compound (PF1198A or -B) was dissolved in acetone (0.25 mL) and then was diluted with 0.25 mL of a 0.1% Tween 20 solution, and the resulting solution was sprayed on the leaf disk. After the evaporation of the acetone, 10 larvae of the aphid were released on the leaf disk and maintained at 25 °C. The mortality was assessed 3 days after this treatment, using 40 larvae for each dose.

Molecular Modeling. Computations were performed using SYBYL, version 6.5, a molecular modeling software (*12*). The starting geometry of IBIPPS was constructed by modifying the X-ray crystal structure of methylbicyclophosphate (*13*), using the SYBYL standard values of bond lengths and angles; the structure was fully optimized by the semiempirical molecular orbital method AM1. The structure of PF1198A was modeled using the SYBYL standard values for bond lengths and angles and was fully optimized in a manner similar to that used for IBIPPS.

RESULTS AND DISCUSSION

Isolation of [^{3}H]EBOB Binding Inhibitors, PF1198A and -B. To discover GABA receptor-directed insecticides in natural products, hundreds of fungal cultures from the Meiji Seika Kaisha collection were screened for their ability to inhibit the specific binding of the radiolabeled noncompetitive antagonist [^{3}H]EBOB to housefly head membranes. As a result, we discovered a fungal strain (PF1198) that produced inhibitors. Starting with 8 kg of the culture of strain PF1198, two inhibitors (111 mg of PF1198A and 56 mg of PF1198B) were isolated by silica gel column and preparative thin-layer chromatography. The fungus producing the inhibitors was identified as *Aspergillus terreus* (see the Supporting Information regarding taxonomy and fermentation).



PF1198A (Alantrypinone): R = H
PF1198B (Serantrypinone): R = OH

Figure 1. Structures of PF1198A and -B isolated from *A. terreus*.

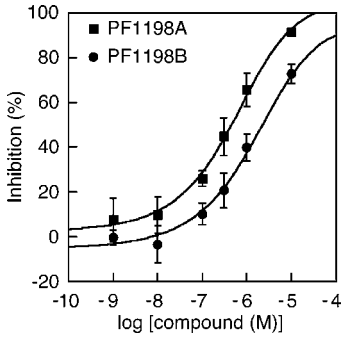


Figure 2. Inhibition of the specific binding of [^{3}H]EBOB to housefly head membranes by PF1198A and -B. The alkaloids dissolved in DMSO (4 μL) at six different concentrations were incubated with housefly head membranes (200 μg of protein) and 0.5 nM [^{3}H]EBOB in 1.0 mL of buffer B at 22 °C for 70 min. The radioactivity of [^{3}H]EBOB that specifically had bound to membranes was measured. Symbols and bars represent means ± SD (n = 3).

Structural Determination of [^{3}H]EBOB Binding Inhibitors. Instrumental analyses (see the Supporting Information for the spectral data) revealed that PF1198A is identical to alantrypinone, which has been isolated as a metabolite of *Penicillium thymicola* (*14*). After we applied for a patent for another inhibitor, PF1198B (*15*), it was reported that *P. thymicola* produced this compound as a minor metabolite, which was then referred to as serantrypinone (*16*). However, the possibility that PF1198B is the enantiomer of serantrypinone cannot be ruled out, since PF1198B and serantrypinone showed specific rotations of opposite signs, i.e., +21.7 and −12° (*16*), respectively. PF1198A is a 3-methyleneoxindole-bridged pyrazoloquinazolinedione alkaloid, and PF1198B is its hydroxy analogue (**Figure 1**). No biological activity has been reported to date for the two alkaloids, although two structurally related alkaloids, spiroquinazoline and fumiquinazoline C, are known as a substance P inhibitor at the human NK-1 receptor (*17*) and a compound showing cytotoxicity toward lymphocytic leukemia cells (*18*), respectively.

Potency of PF1198A and -B as Regards the Inhibition of [^{3}H]EBOB Binding. We performed a series of experiments to determine the potency of PF1198A and -B in terms of their respective abilities to inhibit the specific binding of [^{3}H]EBOB to housefly head and rat brain membranes. As shown in **Figure 2**, PF1198A (IC_{50} = 0.34 μM) inhibited [^{3}H]EBOB binding to the housefly GABA receptor ca. 6-fold more potently than did PF1198B (IC_{50} = 2.1 μM). In the rat GABA receptor, PF1198A (IC_{50} = 16 μM) was 8-fold more potent than PF1198B (IC_{50} = 128 μM) (**Figure 3**). The selectivities that PF1198A and -B showed for housefly vs rat GABA receptors were ca. 47-fold and ca. 61-fold, respectively. The structural difference between the two alkaloids is in the bridgehead substituent, i.e., a methyl group in PF1198A and a hydroxymethyl group in PF1198B. The decrease in the hydrophobicity of the bridgehead substituent

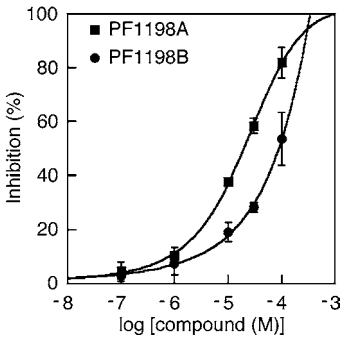


Figure 3. Inhibition of the specific binding of [^{3}H]EBOB to rat brain membranes by PF1198A and -B. The alkaloids dissolved in DMSO (4 μL) at five different concentrations were incubated with rat brain membranes (125 μg of protein) and 0.5 nM [^{3}H]EBOB in 1.0 mL of buffer B at 37 °C for 90 min. The radioactivity of [^{3}H]EBOB that specifically had bound to membranes was measured. Symbols and bars represent means ± SD (n = 3).

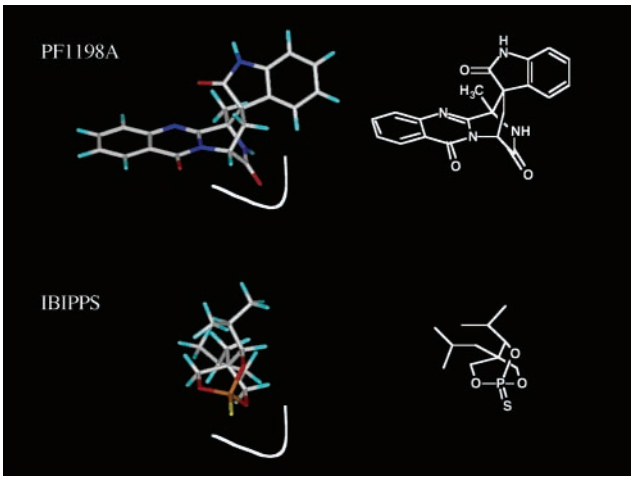


Figure 4. Comparison of the structures of PF1198A and IBIPPS. Note that PF1198A and the known GABA antagonists IBIPPS possess a similar bridged bicyclic moiety as indicated by white curves.

is likely to result in a reduction in the potency of PF1198B. A similar reduction in potency was also observed among bicyclophosphate GABA antagonists (*19*). However, the possibility cannot be ruled out that the larger size of the hydroxymethyl group than the methyl group causes the drop in the potency of PF1198B.

We previously discovered several noncompetitive GABA receptor antagonists that show high selectivity for insect receptors rather than mammalian receptors. Among such compounds is a bicyclophosphorothionate, IBIPPS, which has an IC_{50} value of 45.2 nM for the housefly GABA receptor and shows ca. 53-fold selectivity for housefly vs rat GABA receptors (*20*). In this case, the isopropyl group at the 3-position renders the bicyclophosphorothionate selective. It is of interest to note that both PF1198 and IBIPPS possess a similar bridged bicyclic moiety (**Figure 4**), although it remains to be determined if this partial structural resemblance is responsible for the selective influence of the alkaloids on the insect GABA receptor. Functional assays on the blockage of GABA receptors would also be needed to confirm the results of these binding studies.

Insecticidal Activity of PF1198A and -B. Finally, the alkaloids were examined in terms of their insecticidal activity. A 2:1 mixture of PF1198A and -B did not show insecticidal activity against piperonyl butoxide-pretreated houseflies, when topically applied at a dose of 2 μg/fly. The low penetrability

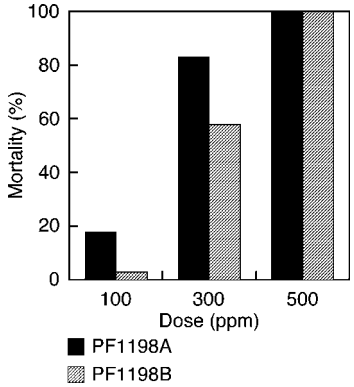


Figure 5. Insecticidal activity of PF1198A and -B against aphids. Leaf disk assays were conducted using the green peach aphid (*M. persicae* Sulzer). Ten larvae were released on a cabbage leaf disk sprayed with a Tween 20 solution containing a test compound at 25 °C. The mortality was assessed 3 days after this treatment, using 40 larvae for each dose (n = 1).

seemed to lower the insecticidal ability of the alkaloids. When injected into piperonyl butoxide-pretreated German cockroaches at doses of 10 and 5.6 μg/roach, the mixture produced 90 and 20% mortality, respectively. In assays using agricultural pest insects, both PF1198A and -B within the range of 100−500 ppm showed significant insecticidal activity against aphids (**Figure 5**). Their LD_{50} values appeared to be between 100 and 300 ppm. Furthermore, PF1198A was found to be more potent than PF1198B in insecticidal assays using aphids, indicating that the insecticidal potency parallels the potency at the receptor.

In conclusion, in the present study, we discovered two insecticidal alkaloids, PF1198A and -B, by employing a binding assay-based screen of fungal culture extracts. The high selectivity of the two alkaloids for insect but not mammalian GABA receptors implied that the two compounds would be less toxic to mammals than to insects. The two alkaloids might be useful leading compounds for the development of highly selective GABA receptor ligands. Binding assay-guided screening using [^{3}H]EBOB is therefore suggested as offering significant opportunities for the identification of novel, safe insecticides.

ABBREVIATIONS USED

GABA, γ-aminobutyric acid; EBOB, 1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane or 4′-ethynyl-4-*n*-propylbicycloorthobenzoate; IBIPPS, 4-isobutyl-3-isopropyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide or isobutyl-(isopropyl)bicyclophosphorothionate.

Supporting Information Available: Spectroscopic data for PF1198A and -B, the descriptions regarding the taxonomy and fermentation of the fungus, and an electron microscopy image of the fungus. This material is available free of charge via the Internet at http://pubs.acs.org.

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