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Original Article

Isocoumarin Derivative as a Novel GABA Receptor Ligand from *Neosartorya quadricincta*

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For the purpose of discovering GABA receptor-directed insecticides in natural products, fungal culture extracts were screened for their ability to inhibit the specific binding of the noncompetitive antagonist [^{3}H]EBOB to housefly head membranes. The screening efforts led to the isolation of a derivative of dihydroisocoumarin (PF1223) from the culture of *Neosartorya quadricincta*. This compound at 2.2 μM inhibited [^{3}H]EBOB binding by 65%. This ligand might prove to be a lead compound for the identification of novel insecticides acting at the insect GABA receptor.

Keywords: GABA receptor, ligand, isocoumarin, *Neosartorya quadricincta*.

INTRODUCTION

The receptor for the inhibitory neurotransmitter GABA is known as a target for such insecticides and acaricides as fipronil and avermectins.[1)] These insecticides selectively act on the insect GABA receptor, and thus are toxic to insects but not mammals. We previously reported that naturally occurring terpenoids such as picrodendrins and anisatin exhibit insecticidal activity by binding to the antagonist site of the GABA receptor.[2–5)] Several terpene lactones such as picrodendrin O exhibited selective affinity for the insect *versus* mammalian GABA receptors. We have also isolated insecticidal alkaloid GABA antagonists from the culture of *Aspergillus terreus* by binding assay-guided screening.[6)] This earlier screening convinced us that our fungal cultures are a rich source of GABAergic or anti-GABAergic compounds. We report here on the subsequent isolation and structural elucidation of a novel ligand for the insect GABA receptor.

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*[1]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; DBCPP, 5-[4-(3,3-dimethylbutoxycarbonyl)phenyl]-4-pentynoic acid.

MATERIALS AND METHODS

1. *Instruments*

The NMR spectra were recorded on a JEOL GSX400 instrument. Mass spectra were obtained on a JEOL JMS-700 spectrometer. The UV and IR spectra were measured on Shimadzu UV-260 and FTIR-8100 spectrophotometers, respectively. Optical rotations were obtained on a JASCO DIP-370 polarimeter. Observations by electron microscopy were performed with a JEOL JSM-6300F electron microscope.

2. *Taxonomic Studies*

Taxonomic studies of strain PF1223 were performed according to the method of Horie *et al.*[7)] The fungal strain PF1223 was deposited at the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Japan, under the accession number FERM P-17658.

3. *Fermentation*

Strain PF1223 was inoculated from an agar slant into a 100-ml Erlenmeyer flask containing 20 ml of a seed medium made up of 1.0% starch, 1.0% glucose, 0.6% wheat germ, 0.2% soybean meal, 0.3% yeast extract, 0.5% polypeptone, 0.2% $CaCO_3$, and tap water (pH 7.0 before sterilization). The inoculated flask was shaken on a rotary shaker (200 rpm) at 25°C

for 3 days. Five milliliters of the seed culture was added to a 500-ml Erlenmeyer flask containing a production medium (pH 7.0, non-adjusted) made up of water-soaked raw rice (100 g) and soybean meal (2.5 g). The inoculated flask was incubated as a stationary phase culture at 25°C for 14 days.

4. Isolation of PF1223

A fungal culture in a rice medium (10 kg) was extracted with 50% acetone in water (20 l). After the extract was filtered and the solvent removed *in vacuo*, the remaining solution (9 l) was extracted with EtOAc (10 l). The extract was concentrated, and 19.05 g of the oily residue was subjected to column chromatography on silica gel (Wakogel C-300, 500 g, Wako; $CHCl_3$/MeOH 10:1). Fractions (1.53 g) that inhibited the binding of [^{3}H]EBOB (Perkin Elmer, 1.11 TBq/mmol) were subjected to Sephadex LH-20 (Pharmacia) gel filtration (eluted with MeOH). Active fractions (873 mg) in [^{3}H]EBOB binding assays were further purified by preparative TLC (silica gel 60 F_{254}, Merck) with $CHCl_3$/MeOH (10:1) to a yield of 10.4 mg of PF1223: $[\alpha]_D^{22.5}$ +11.8° (*c* 0.14, MeOH); UV λ_{max} (MeOH) nm (log ε) 217 (4.28), 264 (4.12), 310 (3.56); UV λ_{max} (0.1 M HCl-MeOH) nm (log ε) 217 (4.20), 264 (4.14), 315 (3.56); UV λ_{max} (0.1 M NaOH-MeOH) nm (log ε) 217 (4.50), 256/s (3.80), 306 (4.17); IR ν_{max} (KBr) cm^{-1} 3410, 2975, 2928, 2855, 1726, 1692, 1651, 1619,1605, 1509, 1466, 1441, 1389, 1371, 1295, 1279, 1248,1208, 1161, 1120, 1078; TSP-MS$^+$ *m/z* 474 $(M+NH_4)^+$; TSP-MS$^-$ *m/z* 455 $(M-H)^-$, 569 $(M+TFA)^-$; FAB-MS$^+$ *m/z* 457 $(M+H)^+$, 479 $(M+Na)^+$; HR-FAB-MS$^+$ *m/z* $(M+Na)^+$: Calcd. for $C_{25}H_{28}NaO_8$: 479.1682, Found: 479.1678.

5. [^{3}H]EBOB Binding Assays

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 a 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 allowed to stand on ice for 30 min. The 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 used directly for the binding assays. For [^{3}H]EBOB binding assays, collected fractions dissolved in MeOH (2 μl) or purified PF1223 dissolved in DMSO (4 μl) were incubated with housefly head membranes (200 μg protein) and 0.5 nM [^{3}H]EBOB in 1.0 ml of Buffer B at 22°C for 70 min. After incubation, the mixtures were filtered through GF/B filters and rapidly rinsed twice with 5 ml of cold (10°C) Buffer B using a Brandel M-24 cell harvester. The radioactivity of [^{3}H]EBOB that specifically bound to membranes on the filters was measured with a liquid scintillation counter. Nonspecific binding was determined in the presence of 5 μM of unlabeled EBOB.

RESULTS AND DISCUSSION

1. Isolation and Structural Determination

For the isolation of GABA receptor ligands, fungal cultures from the Meiji Seika Kaisha collection were screened for their ability to inhibit the specific binding of [^{3}H]EBOB to housefly head membranes. EBOB is a high-affinity ligand that binds to the allosteric antagonist site of the GABA receptor.[8] On performing this screening, we discovered a fungal strain (PF1223) capable of producing an inhibitor. Starting with 10 kg of the culture of strain PF1223, 10.4 mg of an inhibitor was isolated using column chromatography followed by preparative TLC. The inhibitor, isolated as a colorless amorphous powder, gave a molecular-related ion peak at *m/z* 457 for $[M+H]^+$ in the positive-ion FAB-MS. The molecular formula was determined as $C_{25}H_{28}O_8$ based on the detection of an $[M+Na]^+$ ion peak at *m/z* 479.1678 in high resolution FAB-MS.

The spectroscopic data showed that the inhibitor consisted of two moieties: a 3,8-dihydroxy-4,5-dimethyl-4-hydro-3-hydroxymethyl-6-methoxyisocoumarin (Ring A), and a 4-hydroxy-3-prenylbenzoic acid (Ring B) (Table 1). Ring B was attached to the C9 primary hydroxyl group of Ring A *via* an ester deduced as follows. 4-Hydroxy-3-prenylbenzoic acid (Ring B) is a known compound, and is part of the antibiotic novobiocin.[9–13] The structure of Ring B was elucidated by comparing the spectroscopic data of PF1223 with those of 4-hydroxy-3-prenylbenzoic acid. The structure of Ring A was determined mainly from two-dimensional NMR spectra (Fig. 1). The peak at δ 11.2 suggested that a hydrogen-bonded hydroxyl group existed in Ring A. In the HMBC spectrum of PF1223, a benzene ring proton peak at δ 6.39 (H7) showed cross peaks with four benzene ring carbons at δ 99.1 (C8a), 115.5 (C5), 163.1 (C8), and 164.8 (C6). A phenolic hydroxyl proton peak at δ 11.2 exhibited correlation peaks with two carbon signals at δ 97.5 (C7) and 163.1 (C8). A methoxy proton signal at δ 3.87 was also correlated with a carbon signal at δ 164.8 (C6). Furthermore, the signal of methyl protons on the benzene ring was correlated with three carbon signals at δ 164.8 (C6), 115.5 (C5), and 141.4 (C4a). Thus, the benzene ring carbon signals of Ring A were fully assigned. An aliphatic methine proton peak at δ 3.39 (H4) showed correlations with an adjacent methyl carbon signal at δ 16.4 (C4-methyl), three benzene ring carbon signals at δ 141.4 (C4a), 115.5 (C5), and 99.1 (C8a), and a hemiacetal carbon signal at δ 102.4 (C3). Methylene proton signals at δ 4.51 and 4.79 were observed to correlate with one carbonyl carbon signal at δ 166.4 (C7′) and two carbon signals at δ 102.4 (C3) and 36.1 (C4). Consistent with the NMR data, the IR and UV spectra of PF1223 suggested that Ring A was a highly oxygenated dihydroisocoumarin with a structure closely similar to that of sclerotinin A,[14] which was reported to be a plant growth-promoting metabolite from *Sclerotinia sclerotiorum*.

Table 1. NMR spectra data for PF1223

Position	δ $CDCl_3$ (*J*, Hz)		Position	δ $CDCl_3$ (*J*, Hz)	
	^{13}C	^{1}H		^{13}C	^{1}H
1	168.1	—	1′	121.2	—
3	102.4	—	2′	132.2	7.86 m
4	36.1	3.39 q (7.0)	3′	127.2	—
4a	141.4	—	4′	159.4	—
5	115.5	—	5′	115.7	6.86 d (9.0)
6	164.8	—	6′	130.1	7.86 m
7	97.5	6.39 s	7′	166.4	—
8	163.1	—	8′	29.5	3.40 d (7.1)
8a	99.1	—	9′	120.8	5.32 t (7.3)
9	65.9	4.51 d (12.0)	10′	135.8	—
		4.79 d (12.0)	11′	25.8	1.79 s
4-CH_3	16.4	1.28 d (7.1)	12′	17.9	1.79 s
5-CH_3	10.1	2.09 s			
6-OCH_3	55.8	3.87 s			
8-OH	—	11.2 s			

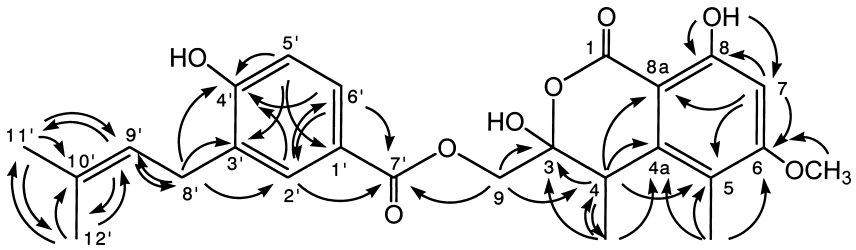

Fig. 1. HMBC correlation for PF1223.

From comparison of the spectroscopic data of PF1223 with those of sclerotinin A, we concluded that Ring A was the 7-demethyl-9-hydroxy-6-*O*-methyl derivative of sclerotinin A. The relationship between Ring A and Ring B was determined by the observation that correlation peaks between the C9 methylene proton signals and the C7′ carbonyl carbon signal were detected in the HMBC measurements described above. The stereochemistry of two chiral carbons at C3 and C4 could not be determined by usual NMR measurements, because the C3 carbon atom lacks an attached hydrogen atom, and as for the C4 carbon atom the adjacent C3 and C4a carbon atoms lack hydrogen atoms. No attempt was made to determine the stereochemistry by X-ray crystallographic analysis, because only a small quantity of PF1223 was available and the activity was not extremely high.

2. *Taxonomy*

The mycological characteristics of strain PF1223 were as follows. The colonies grown on Czapek yeast extract agar at 25°C attained a diameter of 75–85 mm in 14 days, and were yellowish-white, velvety, and composed of compact basal felt. Ascomata and conidiogenesis were produced in abundance. The reverse sides of the colonies were brown. The colonies growing on oatmeal agar at 25°C attained a diameter of 75–80 mm in 14 days, and were pale yellow and granular in appearance, due to the production of numerous ascomata. The exudate appeared as clear, small drops. The reverse sides of the colonies were yellowish-white. The colonies on all media grew more rapidly at 37°C than at 25°C. The ascomata were non-ostiolate, superficial, globose to subglobose, and 50–200 μm in diameter. The peridia were thin and membranous. The asci were borne singly, globose to subglobose, 12–15×10–12 μm, 8-spored, and evanescent at maturity. The ascospores were globose to subglobose and 5–6 μm in diameter, and they had four irregular crests (Fig. 2). The anamorphic strain PF1223 belonged to the section Fumigati in the genus *Aspergillus*. These characteristics indicate that the fungus belongs to *Neosartorya quadricincta*.

3. *Biological Activity*

Finally, we conducted experiments to determine the potency of purified PF1223 in inhibiting the specific binding of [^{3}H]EBOB to housefly head membranes. PF1223 at 2.2 μM inhibited the [^{3}H]EBOB binding by 65%. We previously performed structure-activity studies on straight-chain GABA antagonists and found that butyl benzoates, phenyl pentanoates, benzyl butyl ethers, and pentyl phenyl ethers, which all have an important oxygen atom(s) between hydrophobic moieties,

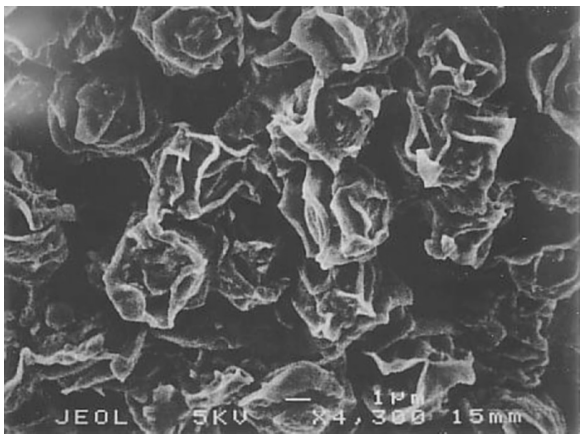

Fig. 2. Electron micrograph of ascospores of *Neosartorya quadricincta* (strain PF1223).

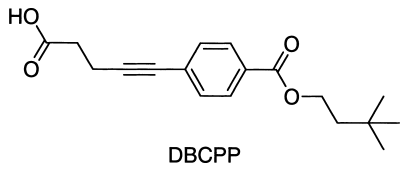

Fig. 3. Structure of the noncompetitive GABA receptor antagonist DBCPP.

act as noncompetitive antagonists for the GABA receptors.[15] The IC_{50} values of DBCPP (Fig. 3), one of the benzoates, in the [^{3}H]EBOB assay were 88 nM and 3.41 μM for the rat and housefly GABA receptors, respectively. Moreover, DBCPP suppressed GABA-induced whole-cell currents in rat dorsal ganglion neurons with an IC_{50} of 0.54 μM. Considering that PF1223 is a benzoate, it is speculated that PF1223 and DBCPP might share a common mode of action, although the structures of these compounds do not necessarily resemble each other. Much more detailed study would be needed before such a conclusion is reached. The potency of PF1223 toward the housefly GABA receptor seems to be in the same range as that of DBCPP. Although PF1223 was expected to be insecticidal, the insecticidal activity was not determined, because only a small quantity of PF1223 was available and the *in vitro* activity was not extremely high. However, this isocoumarin derivative might prove to be a lead compound for the identification of novel insecticides acting at the insect GABA receptor.

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