J. Pestic. Sci., 31(4), 417–419 (2006)
DOI: 10.1584/jpestics.G06-11

Note

Affinity of 3-benzylidene- and 3-cinnamylidenemyosmine analogues for *Periplaneta americana* nicotinic acetylcholine receptors

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(Received April 17, 2006; Accepted June 30, 2006)

3-Benzylidenemyosmine analogues with substituents at the *ortho*-, *meta*-, and *para*-positions of the phenyl ring and 3-cinnamylidenemyosmine analogues with substituents at a *para*-position of the phenyl ring were synthesized. The affinity of the synthesized compounds for nicotinic acetylcholine receptors (nAChRs) in the nerve cord of the American cockroach (*Periplaneta americana* L.) was determined by radioligand binding assay using [^{3}H]epibatidine. Of the compounds tested, 3-(2,4-dihydroxybenzylidene)myosmine **3** and 3-(4-hydroxybenzylidene)myosmine **4** displayed the highest potency, with IC_{50} values of 0.120 and 0.310 μM, respectively. 3-(4-Dimethylaminobenzylidene)myosmine **15** with a *para*-dimethylamino group on the benzylidene moiety displayed moderate affinity (IC_{50}=5.49 μM). On the other hand, cinnamylidene analogues, 3-(4-dimethylaminocinnamylidene)myosmine **16** and 3-cinnamylidenemyosmine **17**, displayed moderate affinity, with IC_{50} values of 2.07 and 3.52 μM, respectively. © Pesticide Science Society of Japan

Keywords: nicotinic acetylcholine receptor, 3-benzylidenemyosmine, American cockroach.

Introduction

Nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels that mediate rapid excitatory neurotransmission, have drawn significant attention as effective targets for insecticides since insect-selective neonicotinoids such as imidacloprid were discovered.[1,2]

3-Benzylideneanabaseine analogues are derived from the marine worm toxin, anabaseine, which is a relatively nonselective mixed agonist/antagonist for multiple nAChR subtypes.[3] 3-(2,4-Dimethoxybenzylidene)anabaseine (GTS-21) has agonist activity at vertebrate $\alpha7$ nAChR and antagonist activity at vertebrate $\alpha4\beta2$ nAChR, although it is unknown to what extent the $\alpha7$ selectivity of GTS-21 results from the addition of the benzylidene moiety and other substituents at the 2- or 4-position.[4–6] While the benzylidene derivation of anabaseine is sufficient to produce selective agonist activity for the $\alpha7$ receptor subtype, 4-substitutions on the benzylidene ring modulate both the agonist and antagonist properties.[6]

We have previously reported that a series of 3-benzylideneanabaseine (BA) analogues and 6′-chloro-3-benzylideneanabaseine (CBA) analogues, which have a chlorine atom at the 6′-position of the pyridine ring, display high affinity for nAChRs of the American cockroach (*Periplaneta americana* L.).[7,8] Structure–activity studies suggested that both the electronic and steric properties of the *para*-substituent of the phenyl group affect the potency of CBA analogues; the greater the electron-donating ability and the smaller the minimum width of the substituents, the higher the potency of these analogues.[8]

Myosmine, which was isolated from *Nicotina tabacum*, is structurally related to anabaseine; myosmine has a pyrrolidine heterocyclic ring, while anabaseine has a piperidine ring.[9]

It has been found that 3-pyridylmethylamine moiety, containing a highly basic nitrogen atom protonated at physiological pH, is the essential moiety for nicotinoid toxicity.[10] We became interested in the affinity of 3-benzylidene- and 3-cinnamylidenemyosmine analogues for insect nAChRs, even if myosmine itself, with low basic nitrogen, has little binding affinity for housefly and honeybee nAChRs.[10]

This report describes the binding activity of 3-benzylidene- and 3-cinnamylidenemyosmine analogues for *P. americana* nAChRs.

Materials and Methods

1. *Materials*

Melting points were measured with a Yanako MP-500D micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured with a JEOL JNM-A400 spectrometer with tetramethylsilane as an internal standard; chemical shifts are expressed as δ values. Mass spectra were determined on a Hitachi M80-B spectrometer.

3-Benzylidenemyosmine analogues were prepared according to the methods described by Zoltewicz *et al.* (Fig. 1).[11] The following procedure for the preparation of 3-(4-hydroxybenzylidene)myosmine **4** is typical.

A mixture of myosmine[12] (0.300 g, 2.05 mmol), 4-hydroxybenzaldehyde (0.580 g, 4.73 mmol), conc. HCl (5 drops) in abs. EtOH (3.5 ml) was heated at 60°C for 2 days. After the solution was cooled to room temperature, the solution was neutralized with sat. $NaHCO_3$ solution and concentrated. The residue was purified by chromatography on silica gel, eluting with

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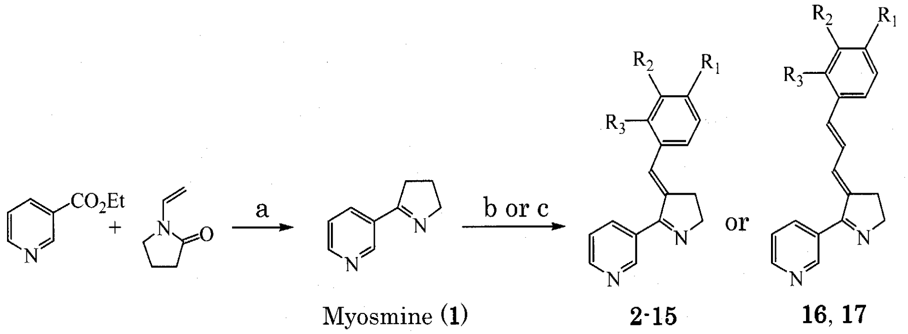


Fig. 1. Synthesis of 3-benzylidene- and 3-cinnamylidenemyosmine analogues. Reagents and conditions: a) i) NaH, THF, reflux, ii) aq. HCl, reflux, b) arylaldehyde, conc. HCl, abs. EtOH, 60°C; c) arylaldehyde or cinnamaldehyde, 0.6 M acetic acid, 0.2 M sodium acetate, abs. MeOH, 60°C.

CH_2Cl_2/MeOH (30 : 1) to give **4** as a yellow solid (0.220 g, 43.5%), mp 180.9–181.7°C. ^{1}H NMR δ (CD_3OD): 8.74 (1H, dd, J=2.2, 0.7 Hz), 8.69 (1H, dd, J=4.9, 1.7 Hz), 8.04 (1H, ddd, J=7.8, 2.2, 1.7 Hz), 7.59 (1H, ddd, J=7.8, 4.9, 0.7 Hz), 7.35 (2H, d, J=8.5 Hz), 6.82 (2H, d, J=8.5 Hz), 6.69 (1H, t, J=2.7 Hz), 4.19–4.22 (2H, m), 3.11–3.14 (2H, m). MS *m/z* (%): 250 (M^+, 100), 118 (43).

2. *Biological assays*

2.1. *Preparation of membranes from P. americana nerve cords*

Membranes from *P. americana* nerve cords were prepared according to the method of Orr *et al.*[13] and modified as described previously.[7] American cockroaches were dissected in ice-cold 50 mM Tris–HCl buffer containing 200 mM sucrose and 1 mM EDTA, pH 7.4 (buffer A). The isolated nerve cords were homogenized on ice with a glass-Teflon homogenizer (25 strokes) in buffer A (2 ml). The homogenate was then centrifuged at 25,000×*g* for 30 min. The supernatant was removed, and the surface of the resulting pellet was washed with buffer A (2 ml). The pellet was then suspended in buffer A (7 ml), and the suspension was centrifuged as above. The supernatant was removed, and the surface of the resulting pellet was washed with buffer A (2 ml) again. The pellet was then suspended in ice-cold 50 mM Tris–HCl containing 120 mM NaCl, pH 7.4 (buffer B) and stored at −80°C. The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard.

2.2 *Binding assays with [^{3}H]epibatidine*

The procedure of this assay is generally based on the method of Orr *et al.*[13] as reported previously.[7] Either a 20-μl aliquot of buffer B for the determination of total binding, a 20-μl aliquot of buffer B containing unlabeled (±)-epibatidine (final conc. 5 μM) for the determination of non-specific binding, or a 20-μl aliquot of buffer B containing a test compound for the determination of inhibitory activity was added to test tubes. Both buffer B (100 μl) containing 2 nM (final conc.) of [^{3}H](±)-epibatidine (Amersham Biosciences UK Limited, 1.96 TBq/mmol) and buffer B (80 μl) containing nerve cord membranes (40 μg protein) were added to all test tubes, which were then incubated at 24°C for 2 hr. Test compounds were formulated in dimethyl sulfoxide (DMSO) and then diluted serially in buffer B. The final concentration of DMSO in the binding experiments was maintained at 0.1%, which did not affect the binding activity of membranes.

The reaction was terminated by rapid filtration under reduced pressure, using a Brandel M-24 cell harvester through Whatman GF/C filters presoaked with buffer B containing 10 mg of BSA/ml for 2 hr. The filters were rapidly washed twice with 2 ml of cold (10°C) buffer B containing 2 mg of BSA/ml. Filter disks were removed and placed in toluene-Methyl Cellosolve-based scintillation fluid, and bound radioactivity was determined using a Beckman LS 6000SE liquid scintillation counter. Each experiment was performed in duplicate and repeated at least twice. IC_{50} values were estimated by the Probit method.

Results and Discussion

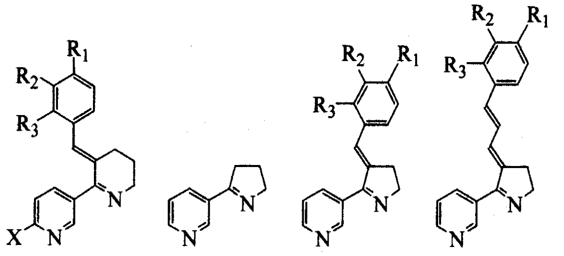
Myosmine (**1**), 3-benzylidenemyosmine analogues (**2**–**15**) and 3-cinnamylidenemyosmine analogues (**16** and **17**) inhibited the specific binding of [^{3}H]epibatidine to *P. americana* nerve cord membranes. IC_{50} values of these compounds are presented in Table 1. Both myosmine and 3-benzylidenemyosmine showed little affinity, regardless of the addition of a benzylidene moiety to the 3-position of myosmine. This finding is in contrast to the previous observation that 3-benzylideneanabaseine displayed high affinity for *P. americana* nAChRs, whereas anabaseine showed little affinity.

Of the compounds tested, analogues (**3** and **4**) with a *para*-hydroxyl group on the benzylidene moiety displayed the highest affinity, with IC_{50} values of 0.120 and 0.310 μM, respectively, for *P. americana* nAChRs. On the other hand, an analogue (**6**) with an *ortho*-hydroxyl group on the benzylidene moiety of 3-benzylidenemyosmine displayed moderate affinity and an analogue (**5**) with a *meta*-hydroxyl group on the benzylidene moiety showed little affinity.

Compounds with a methoxy group of the benzylidene moiety, **9**, **10**, and **11**, also showed little affinity (IC_{50} >10 μM). Analogues (**7**, **8**) having electron-donating *ortho*- and *para*-substituents on the benzene ring displayed moderate affinity.

Analogues with an electron-withdrawing cyano group, **14**, those with a halogenous (chlorine and fluorine) atom at the 4-position of the phenyl group of 3-benzylidenemyosmine, **12** and **13**, showed little affinity.

Table 1. Potencies of BA, CBA, myosmine, 3-benzylidene- and 3-cinnamylidene myosmine analogues in the inhibition of [^{3}H]epibatidine binding to *P. americana* nerve cord membranes



BA (X=H) **Myosmine (1)** **2·15** **16, 17**
CBA (X=Cl)

Compound	R^1	R^2	R^3	IC$_{50}$ (μM)
BA	H	H	H	0.0350 (0.0251–0.0485)$^{b)}$
CBA	H	H	H	0.0212 (0.0146–0.0307)$^{b)}$
1	—	—	—	>10 (27.3)$^{a)}$
2	H	H	H	>10 (41.3)$^{a)}$
3	OH	H	OH	0.120 (0.100–0.144)$^{b)}$
4	OH	H	H	0.310 (0.243–0.395)$^{b)}$
5	H	OH	H	>10 (32.1)$^{a)}$
6	H	H	OH	6.90 (4.90–9.72)$^{b)}$
7	OCH_3	H	OH	3.89 (3.24–4.68)$^{b)}$
8	OCH_3	H	OCH_3	4.55 (3.76–5.50)$^{b)}$
9	OCH_3	H	H	>10 (31.4)$^{a)}$
10	H	OCH_3	H	>10 (23.6)$^{a)}$
11	H	H	OCH_3	>10 (49.5)$^{a)}$
12	F	H	H	>10 (22.3)$^{a)}$
13	Cl	H	H	>10 (21.5)$^{a)}$
14	CN	H	H	>10 (31.4)$^{a)}$
15	$N(CH_3)_2$	H	H	5.49 (4.84–6.24)$^{b)}$
16	$N(CH_3)_2$	H	H	2.07 (1.79–2.38)$^{b)}$
17	H	H	H	3.52 (3.08–4.02)$^{b)}$

[a)] Inhibition % at 10 μM. [b)] 95% confidence limit.

Our data indicate that the introduction of the *para*-hydroxyl group into the phenyl moiety of 3-benzylidenemyosmine increases the binding affinity to *P. americana* nAChRs. One possible cause might be that the addition of the *para*-hydroxyl group to the benzylidene moiety has a sufficient electron-donating effect to increase the negative charge of the imine nitrogen of myosmine by the inductive and resonance effect of the substituents. Furthermore, the results also indicate that occupation of the *ortho*-position with an electron-donating group on the benzylidene moiety of 3-benzylidenemyosmine analogue in the presence of either a hydroxyl or methoxy group as *para*-substituent enhances the affinity for nAChRs.

3-(4-Dimethylaminocinnamylidene)anabaseine was found to be a selective vertebrate α7 nAChR agonist.[4)] To compare the effect of the benzylidene moiety of 3-benzylidenemyosmine with that of cinnamylidene moiety, we performed a binding assay for *P. americana* nAChRs. An analogue, **15**, with the *para*-dimethylamino group of the benzylidene moiety displayed moderate affinity (IC$_{50}$=5.49 μM). On the other hand, cinnamylidene analogues, **16** and **17**, displayed moderate affinity, with IC$_{50}$ values of 2.07 and 3.52 μM, respectively. We anticipated that cinnamylidene analogues may not indicate higher affinity for *P. americana* nAChRs than **2** because of steric hindrance between bulky cinnamylidene moiety and an nAChR binding site; however, **17** showed higher potency than **2** (IC$_{50}$ >10 μM).

Further structure–activity relationship studies of 3-benzylidene- and 3-cinnamylidenemyosmine analogues are now in progress. In addition, it is necessary to examine whether 3-benzylidenemyosmine analogues exhibit insecticidal activity against American cockroaches.

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