Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2963–2971

BIOORGANIC & MEDICINAL CHEMISTRY

Structure–Activity Relationships of Benzylidene Anabaseines in Nicotinic Acetylcholine Receptors of Cockroach Nerve Cords

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Received 19 February 2002; accepted 10 April 2002

Abstract—Ten analogues of 6′-chloro-3-benzylideneanabaseine (CBA) bearing substituents at the *ortho*- and the *para*-positions of the phenyl group were synthesized, together with two related compounds. The affinity of the synthesized compounds for nicotinic acetylcholine receptors (nAChRs) in the nerve cord of the American cockroach (*Periplaneta americana* L.) was examined by the radioligand binding assay using [^{3}H]epibatidine (EPI), a nAChR agonist. All 12 tested compounds inhibited [^{3}H]EPI binding, showing K_i values ranging from 14.6 to 6830 nM. The potency variation of *para*-substituted CBA analogues was explained by the steric (ΔB_1) and electronic (σ_p) parameters of the *para*-substituents, or by the steric parameter and the charge of the N1 nitrogen atom (qN_1). Among the CBA analogues, only two compounds containing a dimethylamino group and a methoxy group at the *para*-position showed high insecticidal activity against the German cockroach (*Blattella germanica*) when injected after pretreatment with metabolic inhibitors. High-affinity analogues of CBA might be suitable probes for use in classifying and characterizing insect nAChR subtypes.

Introduction

The neurotransmitter acetylcholine (ACh) is released from the terminal of the presynaptic neuron, and then binds to the nicotinic acetylcholine receptor (nAChR) on the postsynaptic neuron to mediate fast excitatory neurotransmission between neurons. The nicotinic acetylcholine receptor is a ligand-gated ion channel that is composed of five subunits.[1] ACh and other agonists open the channel and enhance cation permeability, which subsequently depolarizes the membrane. In mammals and other vertebrates, nAChRs are expressed both at the neuromuscular junction (NMJ) and in the nervous system (NS).[2,3] The nAChR at the NMJ contains α1, β1, γ (or ε), and δ subunits, whereas the nAChR in the NS is composed of α2–α10 and β2–β4 subunits.[4,5] On the other hand, insect nAChRs, which are found only in the NS, apparently contain both α-type and non-α-type subunits; for example, in the fruit fly (*Drosophila melanogaster* Meigen), cDNAs encoding four α subunits [ALS, SAD (or Dα2), Dα3, and Dα4] and two non-α subunits (ARD and SBD) have been cloned.[6,7] However, the subunit compositions and stoichiometries of native nAChRs in all insect species remain unknown, and the insect receptor subunits cloned to date do not form a robust functional ligand-gated channel unless coexpressed with a vertebrate β subunit.[8]

Benzylidene anabaseines, which were initially derived from a marine worm toxin anabaseine, have been reported to be selective agonists for α7-nAChR in mammals, and to exhibit antagonist activity at α4β2-nAChR.[9–12] One of the analogues (GTS-21, Fig. 1) is the first drug candidate for the Alzheimer's disease.[13] Our previous electrophysiological study showed that benzylidene anabaseines act as agonists in the nAChRs of the American cockroach (*Periplaneta americana* L.).[14] 3-Benzylideneanabaseine (BA) and 6′-chloro-3-benzylideneanabaseine (CBA) displayed high affinity for *P. americana* nAChRs, while the parent compound anabaseine showed little affinity.[14] The introduction of a nitro group at the *para*-position of the phenyl group led to a decrease in the affinity of both BA and CBA, though the presence of a chlorine atom at the 6′-position of the pyridine ring had little effect on affinity. These findings prompted us to investigate the structure–activity relationships of this class of agonists in *P. americana* nAChRs in more detail, by synthesizing additional analogues of CBA. In the present study, using the nAChR agonist [^{3}H]epibatidine (EPI) as a radioligand, we examined the affinity of CBA analogues for *P. americana* nAChRs and their insecticidal activity against the German cockroach (*Blattella germanica* L.).

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0968-0896/02/$ - see front matter
PII: S0968-0896(02)00139-6

We report here our results, and discuss the interaction of agonists with insect nAChRs.

Results

Chemistry

In the present study, we synthesized 10 analogues (**2**–**11**) of CBA (**1**) and **13** by the reaction of an appropriate aldehyde with 6′-chloroanabaseine, which was obtained from ethyl 6-chloronicotinate and 2-piperidone (Fig. 2, Scheme 1). 5-Benzylidene-6-phenyl-2,3,4-trihydropyridine (**14**) was similarly prepared by the reaction of benzaldehyde with 2-phenyl-2,3,4,5-tetrahydropyridine, which was obtained from ethyl benzoate and 2-piperidone.

Potency of benzylidene anabaseines and related compounds in *P. americana* nAChRs

All tested compounds inhibited specific [^{3}H]EPI binding to *P. americana* nerve cord membranes in the nanomolar to micromolar range (Table 1). Figure 3A and 3B show the dose-dependence of the inhibition of [^{3}H]EPI binding by CBA analogues bearing an electron-donating group(s) at the *para*- and the *ortho*-positions of the phenyl group. Based on the IC_{50} values estimated from the dose-response data, we calculated the Cheng–Prusoff's inhibition constant K_i for each compound. Of the six compounds tested, **3** ($K_i = 18.2$ nM) and **4** ($K_i = 14.6$ nM), with a hydroxy group(s), demonstrated the highest potency to inhibit [^{3}H]EPI binding. The affinity of **5** ($K_i = 36.4$ nM) and **6** ($K_i = 192$ nM), bearing a methoxy group(s), for *P. americana* nAChRs was lower than that of **3** or **4**. The introduction of a methoxy group at the *ortho*-position decreased the potency of **5**, indicating that the sterical bulkiness of this substituent might be unfavorable. Compound **2**, with a methyl group at the *para*-position, displayed moderate potency, with a K_i of 259 nM. Compound **7**, with a dimethylamino group at the *para*-position, showed lower potency ($K_i = 56.1$ nM) than that of **3** or **4**, and higher potency than that of **2** or **6**. Figure 3C shows the dose-response curves of analogues containing an electron-withdrawing group at the *para*-position of the phenyl group. These compounds, excepting a fluoro analogue (**8**) with a moderate potency ($K_i = 306$ nM), demonstrated micromolar potency to inhibit [^{3}H]EPI binding to *P. americana* nAChRs (Table 1).

To investigate the effect of replacement of the benzylidene moiety with a different group, we performed a binding assay of the cinnamylidene congener (**13**) of CBA (**1**), which retained potency but was approximately 14-fold less potent ($K_i = 292$ nM) than CBA (Fig. 3D, Table 2). To determine the importance of the nitrogen atom on the pyridine ring, we tested a compound (**14**) that had a benzene ring instead of a pyridine ring. Compound **14** ($K_i = 371$ nM) was approximately 18-fold less potent than CBA (**1**), indicating that the presence of the nitrogen atom on the pyridine ring is important, possibly as a hydrogen bond acceptor, but not crucial, which finding was in accord with observations on other nicotinic ligands.[15]

Quantitative structure–activity relationship (QSAR) analysis

To determine which factors govern the variation in potency, regression analysis was performed for the pK_is of CBA (**1**) and nine mono-substituted CBA analogues

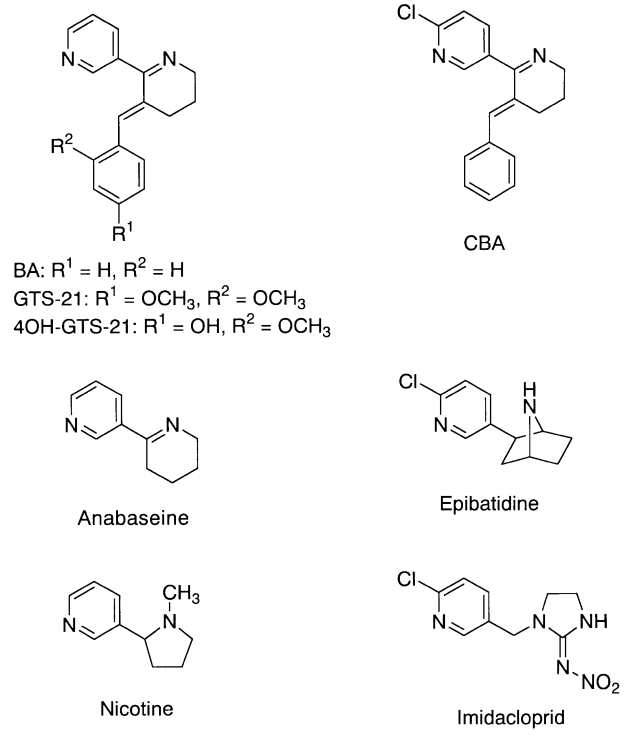


Figure 1. Structures of nAChR agonists.

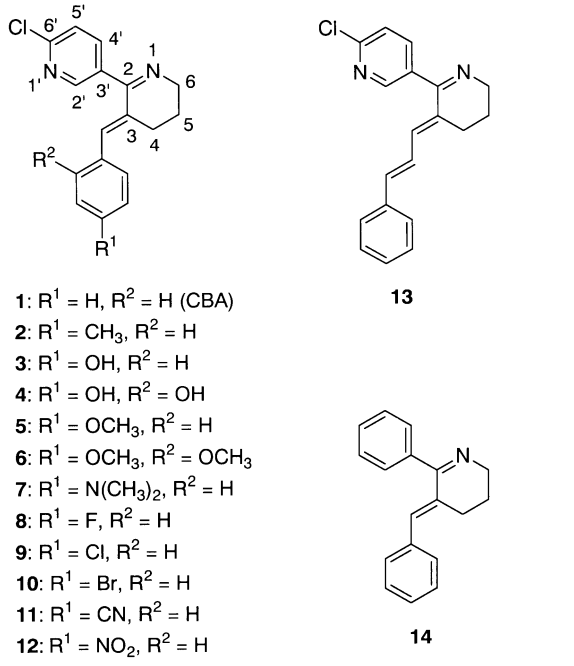
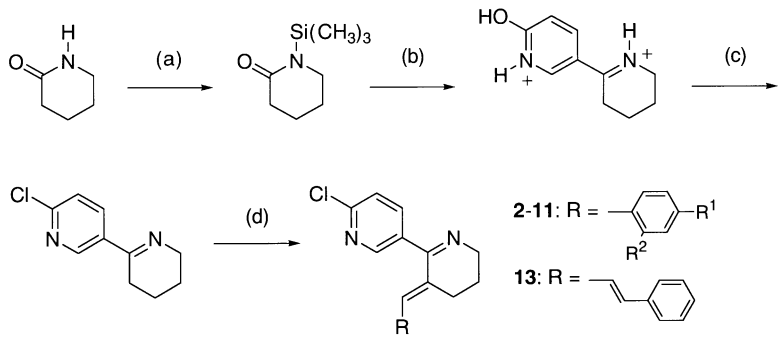


Figure 2. Structures of synthesized benzylidene anabaseines and related compounds.



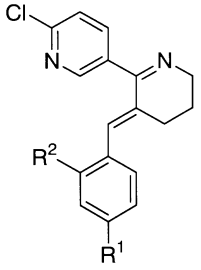
Scheme 1. Reagents: (a) LDA, Me_3SiCl; (b) (1) ethyl 6-hydroxynicotinate; (2) HCl; (c) $POCl_3$; (d) RCHO, AcOH, AcONa.

(**2**, **3**, **5**, **7**–**12**), using various parameters.[16] The following equation was derived using the electronic parameter σ_p and the steric parameter ΔB_1 of the *para*-substituents:

$$pK_i = -0.96(\pm 0.28)\sigma_p - 2.60(\pm 0.49)\Delta B_1 + 7.75(\pm 0.26)$$
$$n = 10,\ \ s = 0.35,\ \ r = 0.96,\ \ F_{2,7} = 39.51 \qquad (1)$$

In this and the following equation, n is the number of compounds included in each equation; s, the standard deviation; r, the correlation coefficient; and F, the F value of the correlation. B_1 is a STERIMOL parameter[17] defined as the minimum width of the substituent from the axis connecting the α-atom of the substituent with the rest of the molecule. The ΔB_1 scale is based on $H = 0$. Table 3 lists the pK_i values and the physicochemical parameters used to derive the equation. Figure 4 displays a good correlation between the observed and the calculated pK_i values. Eq (1) indicates that the potency of the compounds decreases with an increase in the electron-withdrawing ability of the substituents, and that the minimum width of the *para*-substituent of the phenyl group is a crucial factor for the binding of the compounds to *P. americana* nAChRs.

Table 1. Potency of CBA analogues to inhibit specific [^{3}H]EPI binding to nerve cord membranes of American cockroaches and their insecticidal activity against German cockroaches



Compound	R^1	R^2	[^{3}H]EPI binding	Insecticidal activity
			K_i (nM)	Mortality (%) at 10 μg
1 (CBA)	H	H	21.2 (14.6–30.7)[a,b]	33.3 ± 15.3[c]
2	CH_3	H	259 (198–340)[a]	10.0 ± 10.0[c]
3	OH	H	18.2 (15.7–21.1)[a]	20.0 ± 10.0[c]
4	OH	OH	14.6 (10.8–19.5)[a]	10.0 ± 0.0[c]
5	OCH_3	H	36.4 (26.6–49.7)[a]	66.7 ± 5.8[c,d]
6	OCH_3	OCH_3	192 (171–216)[a]	10.0 ± 0.0[c]
7	$N(CH_3)_2$	H	56.1 (44.1–71.1)[a]	96.7 ± 5.8[c,e]
8	F	H	306 (226–414)[a]	13.3 ± 5.8[c]
9	Cl	H	6500 (4640–9100)[a]	23.3 ± 15.3[c]
10	Br	H	6830 (3910—11,900)[a]	10.0 ± 0.0[c]
11	CN	H	4750 (3240–6950)[a]	3.3 ± 5.8[c]
12	NO_2	H	3730 (2950–4720)[a,b]	13.3 ± 5.8[c]

[a]95% confidence limits are given in parentheses.
[b]Taken from ref 14.
[c]Mean ± standard deviation ($n = 3$).
[d]$LD_{50} = 8.43$ (7.46–9.52) μg/roach.
[e]$LD_{50} = 2.68$ (2.21–3.24) μg/roach.

Eq (2), shown below, was formulated on the basis of the charge (qN_1) of the imine nitrogen atom, which was calculated by the semi-empirical molecular orbital method AM1. As shown in Figure 5, the aromatic rings of the CBA molecule thus constructed were twisted out of the approximate plane of the tetrahydropyridine ring, which finding is in accord with the results of the earlier calculation for *para*-chloro BA.[18]

$$pK_i = -88.44(\pm 37.41)qN_1 - 2.66(\pm 0.61)\Delta B_1 - 6.01(\pm 5.99)$$
$$n = 10,\ \ s = 0.42,\ \ r = 0.94,\ \ F_{2,7} = 25.42 \qquad (2)$$

Eq (2) indicates that the activity of the compounds decreases as the charge of the nitrogen atom becomes less negative. Thus, the results prove that the electronic effects of the *para*-substituents are reflected in the characteristics of the N1 nitrogen atom.

Insecticidal activity

On the basis of the fact that nAChR agonists such as nicotine and imidacloprid (Fig. 1) exhibit insecticidal activity,[19] we investigated the insecticidal activity of all synthesized compounds by injecting them into adult male German cockroaches. Compounds containing a methoxy group (**5**) and a dimethylamino group (**7**) at the *para*-position showed the highest insecticidal activity among the CBA analogues; their LD_{50} values were estimated to be 8.43 and 2.68 μg/cockroach (Table 1), respectively, based on the dose–mortality relationships shown in Figure 6. CBA was almost equipotent to (−)nicotine, which gave about 37% mortality at a dose of 10 μg. Compounds with an electron-withdrawing

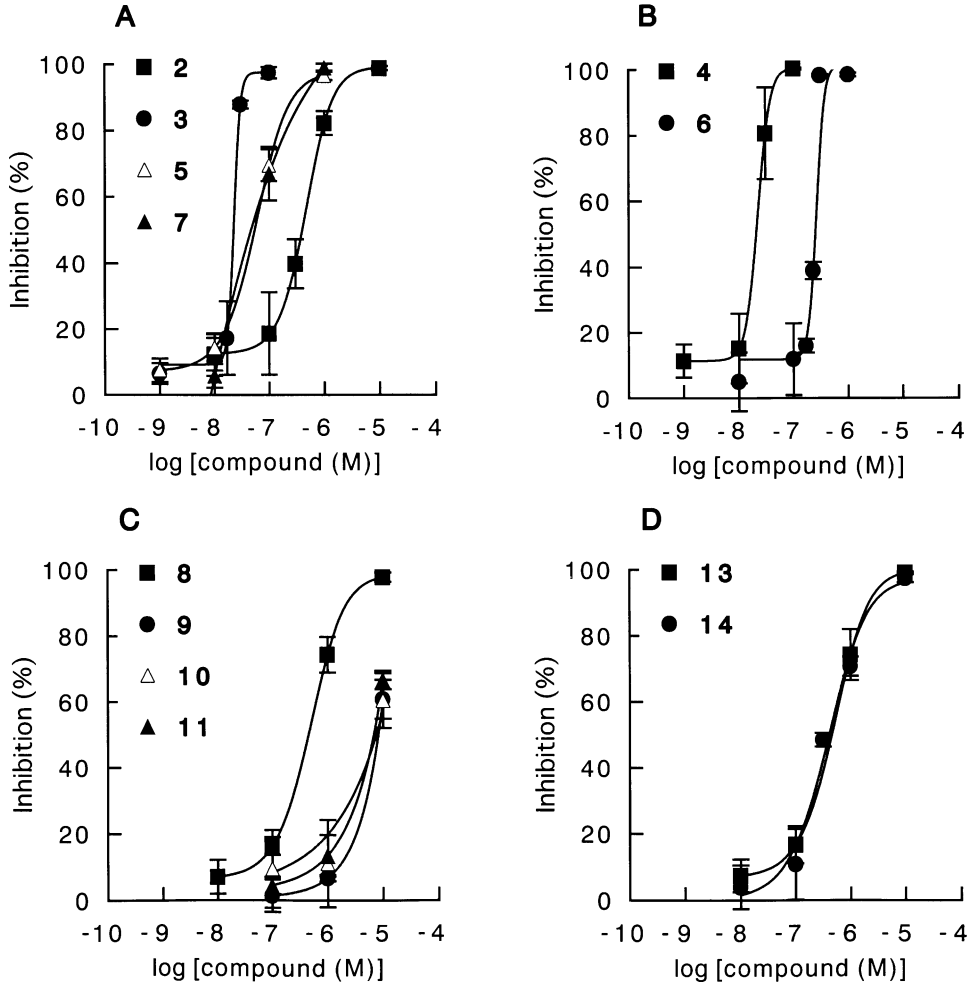


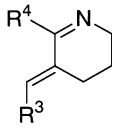
Figure 3. Dose–response curves of benzylidene anabaseines and related compounds in the inhibition of specific [^{3}H]EPI binding to *P. americana* nerve cord membranes. Membrane-bound radioactivity was measured after the incubation of membranes with [^{3}H]EPI at 24 °C for 2 h in the presence and absence of a test compound. Nonspecific binding was determined in the presence of 5 μM unlabeled (±)-EPI. Specific binding was calculated as the difference between total and nonspecific binding. Data are the mean ± standard deviation of three experiments, each done in duplicate, and fitted to the logistic equation.

group showed low insecticidal activities; these compounds exhibited comparatively lower affinity for *P. americana* nAChRs. However, other compounds, such as **3** and **4**, showed unexpectedly low insecticidal activities, although these compounds with an electron-donating group(s) exhibited high affinity.

Discussion

We demonstrated that benzylidene anabaseines act as agonists in cockroach (*P. americana*) nAChRs. In mammalian nAChRs, benzylidene anabaseines are agonists for homooligomeric α7-nAChR but behave as

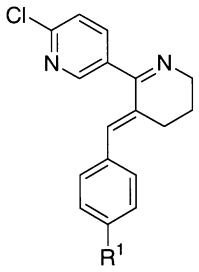
Table 2. Potency of cinnamylidene anabaseine and benzylidene phenyl trihydropyrididne to inhibit specific [^{3}H]EPI binding to nerve cord membranes of American cockroaches and their insecticidal activity against German cockroaches



Compound	R^3	R^4	[^{3}H]EPI binding	Insecticidal activity
			K_i (nM)	Mortality (%) at 10 μg
13	$C_6H_5CH{=}CH$	6′-Cl–3′-C_6H_3N	292 (214–398)[a]	10.0 ± 0.0
14	C_6H_5	C_6H_5	371 (293–469)[a]	30.0 ± 10.0

[a]95% confidence limits are given in parentheses.

Table 3. Regression analysis of structure–activity relationships of 6′-chloro-3-(4-substituted benzylidene)anabaseines



Compound	R^1	σ_p	qN_1	ΔB_1	pK_i		
					Obsd	Calcd	
						Eq 1	Eq 2
1	H	0.00	−0.158	0.00	7.67	7.75	7.97
2	CH_3	−0.17	−0.159	0.52	6.59	6.57	6.67
3	OH	−0.37	−0.158	0.35	7.74	7.20	7.04
5	OCH_3	−0.27	−0.159	0.35	7.44	7.10	7.12
7	$N(CH_3)_2$	−0.83	−0.161	0.35	7.25	7.64	7.30
8	F	0.06	−0.155	0.35	6.51	6.79	6.77
9	Cl	0.23	−0.155	0.80	5.19	5.46	5.57
10	Br	0.23	−0.154	0.95	5.17	5.07	5.08
11	CN	0.66	−0.151	0.60	5.32	5.56	5.75
12	NO_2	0.78	−0.146	0.70	5.43	5.19	5.04

antagonists for heterooligomeric α4β2-nAChR.[10–13] In this respect, *P. americana* nAChRs that responded to benzylidene anabaseines resemble α7-nAChR. The CBA analogues (**3**, **4**) with a hydroxy group(s) at the *para*-position of the phenyl group displayed the highest affinity for *P. americana* nAChRs, showing K_is of ca. 15–18 nM. These compounds are 16- to 20-fold more potent than (−)nicotine and only 2- to 3-fold less potent than the nicotinic agonist insecticide imidacloprid; in the same assay system, the K_is of (−)nicotine and imidacloprid were reported to be 496 and 5.95 nM, respectively.[20] GTS-21, an α7-nAChR agonist drug candidate, inhibited the specific binding of the α7-nAChR ligand [^{125}I]α-bungarotoxin to rat PC12 cell membranes ($K_i = 0.31$ μM) and to human SK-N-SH cell membranes ($K_i = 23$ μM).[11] The affinity of the 6′-chloro analogue (**6**) of GTS-21 for *P. americana* nAChRs is apparently comparable to that of GTS-21 in rat α7-nAChR, although the radioligands used are different. *para*-Desmethyl GTS-21 (4OH-GTS-21, Fig. 1), the major metabolite of GTS-21 in humans, showed K_is of 0.17 and 0.45 μM in PC12 and SK-N-SH cell membranes, respectively. The observation that the potency of the 4-hydroxy analogue is higher than that of the 4-methoxy analogue in human SK-N-SH cell membranes is the same as that in the potencies of **4** and **6** in *P. americana* nerve cord membranes.

Nicotine is an agonist in both vertebrate and invertebrate nAChRs.[19] However, neonicotinoids such as imidacloprid have been reported to act as selective agonists for insect versus vertebrate nAChRs. At the pH of the insect body fluid, the basic nitrogen atom on the pyrrolidine ring of nicotine is protonated as a one-unit positively charged center, which is one of the sites of interaction with the receptor, as is the case in the quaternary nitrogen atom of ACh. In contrast, the corresponding nitrogen atom of neonicotinoids bears a partial positive charge, which is conferred by a neighboring electron-withdrawing group such as a nitro or a cyano group.[21] According to Yamamoto,[19] neonicotinoids are supposed to interact with insect nAChRs, but not with their vertebrate counterparts, due to this partial positive charge of the amine nitrogen atom on the imidazolidine ring. On the other hand, it was proposed that the negatively charged tip of the nitro group of imidacloprid is important for selective interaction with insect nAChRs.[22–24] Matsuda et al.[7] suggested that the interaction of amino acid residues of insect nAChRs with the nitro group enhances the partial positive charge of the tertiary amine nitrogen atom to a level comparable to that of the quaternary nitrogen atom of ACh. The selectivity of neonicotinoid insecticides for insect versus vertebrate nAChRs implies that there might be structural differences in the agonist binding site between animal species. The electron-withdrawing *para*-substituents of CBA analogues could be expected, then, to exert effects similar to those of the nitro group of imidacloprid on the imine nitrogen atom; that is, they might make the imine nitrogen atom partially positive. However, the situation differed between the two types of compounds; the electron-withdrawing *para*-substituents of CBA analogues slightly decreased the negative charge of the imine nitrogen atom, and thereby resulted in a reduction in affinity for *P. americana* nAChRs (Table 3).

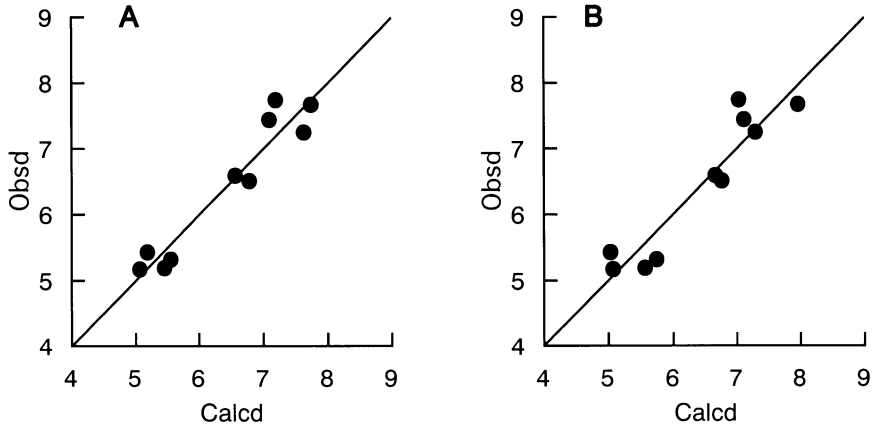


Figure 4. Relation between observed and calculated pK_i values: (A) eq 1; (B) eq 2.

The results of QSAR analysis revealed that two factors affect the binding of *para*-substituted CBA analogues to *P. americana* nAChRs; the electronic and steric properties of the substituents. One of the two factors is the inductive and resonance effect of the substituents. The electron-withdrawing *para*-substituents reduce the electron density of the imine nitrogen atom, which is important in interaction with nAChRs, through the conjugated double bonds. A similar relationship between the pK_i values and the imine nitrogen basicity (pK_a) of benzylidene anabaseines has been reported, evidenced by their inhibition of [^{3}H]methyl carbamylcholine binding to rat brain membranes.[25] The present study revealed the minimum width of the substituents to be another important factor affecting affinity of the analogues for *P. americana* nAChRs. A portion of the space around the substituent, though not the whole space, could be restricted in the binding site.

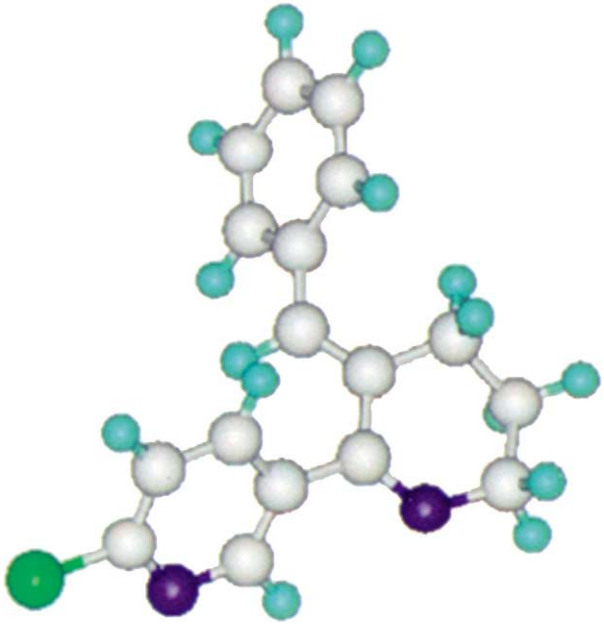


Figure 5. 6′-Chloro-3-benzylideneanabaseine modeled by the AM1 calculation.

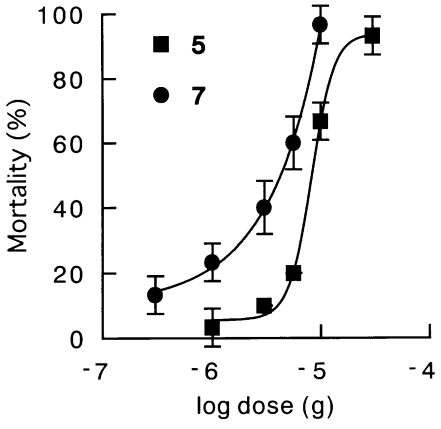


Figure 6. Dose–mortality curves of **5** and **7** in insecticidal assays using German cockroaches.

Finally, we measured the insecticidal activity of the synthesized compounds by injecting them into German cockroaches. Of the 14 analogues tested, only two compounds with high activity in vitro showed high insecticidal activity, with LD_{50}s of $<10\,\mu g$/cockroach. However, these compounds were estimated to be approximately 8- and 25-fold less active than imidacloprid ($LD_{50} = 21.7$ nmol/g[20]). Other 12 analogues tested showed only low insecticidal activity. Appropriate hydrophobicity of compounds is an important factor in their penetration into the central NS of insects. The hydrophilic, readily ionizable properties of benzylidene anabaseines might reduce their penetrability to the binding site. Alternatively, they might be transformed into amine or other inactive forms in the body of the cockroach.

Conclusion

In the current study, we showed that CBA analogues such as **1**, **3**, and **4** have a high affinity for cockroach (*P. americana*) nAChRs. Both the electronic and the 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 the analogues. A higher negative charge of the imine nitrogen atom results in a high affinity. The high-affinity agonists found here might prove to be ligands useful in the classification and characterization of insect nAChR subtypes.

Experimental

Chemistry

General. Melting points were determined with a Yanako MP-500D apparatus and are uncorrected. ^{1}H NMR spectra were measured in DMSO-d_6, using tetramethylsilane as an internal standard on a JEOL JNM-A400 (400 MHz) instrument. Mass spectra were obtained at 70 eV on a Hitachi M-80B spectrometer. General chemicals were purchased from Wako Pure Chemical Industries, Ltd., except (±)-EPI and piperonyl butoxide (PB), which were purchased from Sigma-Aldrich Co. and Tokyo Chemical Industry Co., Ltd., respectively. [^{3}H](±)-EPI (1250.6 GBq/mmol) was obtained from NEN Life Science Products, Inc. Propyl 2-propynyl phenylphosphonate (NIA16388) was a gift from Prof. K. Nishimura, Osaka Prefecture University, Osaka, Japan.

6′-Chloro-3-(4-methylbenzylidene)anabaseine (2). 4-Methyl benzaldehyde (280 mg, 2.34 mmol) and 6′-chloroanabaseine (CANA)[14] (227 mg, 1.17 mmol) were added to an aqueous solution (20 mL) containing 0.6 M acetic acid and 0.2 M sodium acetate with stirring, and the mixture was heated at 60 °C for 48 h. After cooling, the solution was made acidic with 5% HCl and washed with EtOAc to remove residual benzaldehyde. The solution was then made alkaline with a saturated $NaHCO_3$

solution and extracted with EtOAc. The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated to give a solid mass. The crude solid was purified by silica-gel column chromatography (*n*-hexane/EtOAc = 2:1) followed by recrystallization from *n*-hexane/EtOAc to afford **2** (128 mg) as a white solid: yield 37%; mp 107.6–108.6 °C; ^{1}H NMR δ 1.70 (q, J = 6.1 Hz, 5-CH_2, 2H), 2.30 (s, CH_3, 3H), 2.76 (t, J = 6.1 Hz, 4-CH_2, 2H), 3.74 (t, J = 6.1 Hz, 6-CH_2, 2H), 6.54 (s, = CH, 1H), 7.19 (d, J = 8.1 Hz, Ph, 2H), 7.29 (d, J = 8.1 Hz, Ph, 2H), 7.55 (d, J = 8.3 Hz, 5′-CH, 1H), 7.91 (dd, J = 2.4, 8.3 Hz, 4′-CH, 1H), 8.46 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{18}H_{17}ClN_2$ (M^+) 296.1080, found 296.1077.

6′-Chloro-3-(4-hydroxybenzylidene)anabaseine (3). The title compound was obtained from 4-hydroxybenzaldehyde and CANA, as described for **2**. **3**: a light yellow solid; yield 36%; mp 211.8–212.0 °C; ^{1}H NMR δ 1.71 (q, J = 5.7 Hz, 5-CH_2, 2H), 2.76 (t, J = 5.7 Hz, 4-CH_2, 2H), 3.71 (t, J = 5.7 Hz, 6-CH_2, 2H), 6.47 (s, = CH, 1H), 6.77 (d, J = 8.7 Hz, Ph, 2H), 7.25 (d, J = 8.7 Hz, Ph, 2H), 7.54 (d, J = 8.3 Hz, 5′-CH, 1H), 7.89 (dd, J = 2.6, 8.3 Hz, 4′-CH, 1H), 8.44 (d, J = 2.6 Hz, 2′-CH, 1H), 9.76 (s, OH, 1H); HRMS m/z (M^+) calcd for $C_{17}H_{15}ClN_2O$ 298.0873, found 298.0865.

6′-Chloro-3-(2,4-dihydroxybenzylidene)anabaseine (4). The title compound was obtained from 2,4-dihydroxybenzaldehyde and CANA, as described for **2**. **4**: an orange solid; yield 46%; mp 163.1–166.4 °C (dec); ^{1}H NMR δ 1.70 (q, J = 5.4 Hz, 5-CH_2, 2H), 2.70 (t, J = 5.4 Hz, 4-CH_2, 2H), 3.73 (br, 6-CH_2, 2H), 6.26–6.30 (m, Ph, 1H), 6.67 (s, = CH, 1H), 7.20 (d, J = 8.3 Hz, Ph, 1H), 7.55 (d, J = 8.1 Hz, 5′-CH, 1H), 7.88 (dd, J = 2.2, 8.1 Hz, 4′-CH, 1H), 8.43 (d, J = 2.2 Hz, 2′-CH, 1H), 9.56 (s, OH, 1H), 9.59 (s, OH, 1H); HRMS m/z (M^+) calcd for $C_{17}H_{15}ClN_2O_2$ 314.0822, found 314.0846.

6′-Chloro-3-(4-methoxybenzylidene)anabaseine (5). The title compound was obtained from 4-methoxybenzaldehyde and CANA, as described for **2**. **5**: a cream-colored solid; yield 43%; mp 78.4–78.6 °C; ^{1}H NMR δ 1.71 (q, J = 6.1 Hz, 5-CH_2, 2H), 2.77 (dt, J = 2.0, 6.1 Hz, 4-CH_2, 2H), 3.73 (t, J = 6.1 Hz, 6-CH_2, 2H), 3.77 (s, OCH_3, 3H), 6.54 (s, = CH, 1H), 6.96 (d, J = 8.8 Hz, Ph, 2H), 7.36 (d, J = 8.8 Hz, Ph, 2H), 7.55 (d, J = 8.3 Hz, 5′-CH, 1H), 7.91 (dd, J = 2.4, 8.3 Hz, 4′-CH, 1H), 8.45 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{18}H_{17}ClN_2O$ 312.1029, found 312.1032.

6′-Chloro-3-(2,4-dimethoxybenzylidene)anabaseine (6). The title compound was obtained from 2,4-dimethoxybenzaldehyde and CANA, as described for **2**. **6**: a yellow solid; yield 35%; mp 118.5–119.3 °C; ^{1}H NMR δ 1.70 (q, J = 5.9 Hz, 5-CH_2, 2H), 2.68 (t, J = 5.9 Hz, 4-CH_2, 2H), 3.70 (s, OCH_3, 3H), 3.76 (t, J = 5.9 Hz, 6-CH_2, 2H), 3.79 (s, OCH_3, 3H), 6.55–6.60 (m, Ph, 2H), 6.61 (s, = CH, 1H), 7.32 (d, J = 8.3 Hz, Ph, 1H), 7.56 (d, J = 8.1 Hz, 5′-CH, 1H), 7.90 (dd, J = 2.4, 8.1 Hz, 4′-CH, 1H), 8.45 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{19}H_{19}ClN_2O_2$ 342.1135, found 342.1150.

6′-Chloro-3-(4-dimethylaminobenzylidene)anabaseine (7). The title compound was obtained from 4-dimethylaminobenzaldehyde and CANA, as described for **2**. The product was treated with HCl and 2-propanol, and the resulting solid mass was recrystallized from methanol/2-propanol to afford the dihydrochloride as a red solid: yield 40%; mp 121.2–123.7 °C (dec); ^{1}H NMR δ 2.03 (q, J = 5.8 Hz, 5-CH_2, 2H), 2.95 (t, J = 5.8 Hz, 4-CH_2, 2H), 3.06 (s, $N(CH_3)_2$, 6H), 3.75 (t, J = 5.8 Hz, 6-CH_2, 2H), 6.79 (d, J = 9.1 Hz, Ph, 2H), 7.12 (s, = CH, 1H), 7.59 (d, J = 9.1 Hz, Ph, 2H), 7.79 (d, J = 8.3 Hz, 5′-CH, 1H), 8.07 (dd, J = 2.4, 8.3 Hz, 4′-CH, 1H), 8.62 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{19}H_{20}ClN_3$ 325.1346, found 325.1370.

6′-Chloro-3-(4-fluorobenzylidene)anabaseine (8). The title compound was obtained from 4-fluorobenzaldehyde and CANA, as described for **2**. **8**: a white solid; yield 56%; mp 87.4–87.8 °C; ^{1}H NMR δ 1.72 (q, J = 6.1 Hz, 5-CH_2, 2H), 2.75 (dt, J = 2.0, 6.1 Hz, 4-CH_2, 2H), 3.75 (t, J = 6.1 Hz, 6-CH_2, 2H), 6.60 (s, = CH, 1H), 7.22 (d, J = 7.8 Hz, Ph, 1H), 7.24 (d, J = 7.8 Hz, Ph, 1H), 7.47 (d, J = 7.8 Hz, Ph, 1H), 7.50 (d, J = 7.8 Hz, Ph, 1H), 7.56 (d, J = 8.3 Hz, 5′-CH, 1H), 7.93 (dd, J = 2.4, 8.3 Hz, 4′-CH, 1H), 8.48 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{17}H_{14}ClFN_2$ 300.0830, found 300.0830.

6′-Chloro-3-(4-chlorobenzylidene)anabaseine (9). The title compound was obtained from 4-chlorobenzaldehyde and CANA, as described for **2**. **9**: a white solid; yield 69%; mp 109.4–109.6 °C; ^{1}H NMR δ 1.72 (q, J = 6.2 Hz, 5-CH_2, 2H), 2.75 (dt, J = 1.9, 6.2 Hz, 4-CH_2, 2H), 3.75 (t, J = 6.2 Hz, 6-CH_2, 2H), 6.59 (s, = CH, 1H), 7.44 (2s, Ph, 4H), 7.56 (d, J = 8.3 Hz, 5′-CH, 1H), 7.93 (dd, J = 2.3, 8.3 Hz, 4′-CH, 1H), 8.48 (d, J = 2.3 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{17}H_{14}Cl_2N_2$ 316.0534, found 316.0486.

6′-Chloro-3-(4-bromobenzylidene)anabaseine (10). The title compound was obtained from 4-bromobenzaldehyde and CANA, as described for **2**. **10**: a white solid; yield 30%; mp 119.5–119.8 °C; ^{1}H NMR δ 1.71 (q, J = 6.2 Hz, 5-CH_2, 2H), 2.74 (dt, J = 2.0, 6.2 Hz, 4-CH_2, 2H), 3.75 (t, J = 6.2 Hz, 6-CH_2, 2H), 6.57 (s, = CH, 1H), 7.35 (d, J = 8.5 Hz, Ph, 2H), 7.56 (d, J = 8.2 Hz, 5′-H, 1H), 7.58 (d, J = 8.5 Hz, Ph, 2H), 7.93 (dd, J = 2.5, 8.2 Hz, 4′-CH, 1H), 8.48 (d, J = 2.5 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{17}H_{14}BrClN_2$ 360.0029, found 360.0135.

6′-Chloro-3-(4-cyanobenzylidene)anabaseine (11). The title compound was obtained from 4-cyanobenzaldehyde and CANA, as described for **2**. **11**: a white solid; yield 53%; mp 140.1–142.6 °C; ^{1}H NMR δ 1.71 (q, J = 5.9 Hz, 5-CH_2, 2H), 2.75 (t, J = 5.9 Hz, 4-CH_2, 2H), 3.78 (t, J = 5.9 Hz, 6-CH_2, 2H), 6.66 (s, = CH, 1H), 7.55 (d, J = 8.4 Hz, 5′-H, 1H), 7.58 (d, J = 8.3 Hz, Ph, 2H), 7.83 (d, J = 8.3 Hz, Ph, 2H), 7.94 (d, J = 8.4 Hz, 4′-CH, 1H), 8.49 (s, 2′-CH, 1H); HRMS calcd for $C_{18}H_{14}ClN_3$ m/z (M^+) 307.0876, found 307.0875.

6′-Chloro-3-(cinnamylidene)anabaseine (13). The title compound was obtained from cinnamaldehyde and CANA, as described for **2**. The product was treated

with HCl and 2-propanol, and the resulting solid mass was recrystallized from methanol/2-propanol to afford the dihydrochloride as a light green solid: yield 27%; mp 150.2–157.4 °C (dec); ^{1}H NMR δ 2.05 (q, J = 5.7 Hz, 5-CH_2, 2H), 2.93 (t, J = 5.7 Hz, 4-CH_2, 2H), 3.79 (t, J = 5.7 Hz, 6-CH_2, 2H), 7.05 (d, J = 11.2 Hz, =CH, 1H), 7.34–7.72 (m, =CH, Ph, 7H), 7.84 (d, J = 8.3 Hz, 5′-CH, 1H), 8.11 (dd, J = 2.4, 8.3 Hz, 4′-CH, 1H), 8.65 (d, J = 2.4 Hz, 2′-CH, 1H); HRMS m/z (M^+) calcd for $C_{19}H_{17}ClN_2$ 308.1080, found 308.1074.

6-Phenyl-2,3,4,5-tetrahydropyridine (PTP). Diisopropylamine (6.0 mL, 45 mmol) and 1.6 M *n*-butyllithium/hexane (30 mL, 48 mmol) were added to dry THF (21 mL) with stirring at −70 °C under a nitrogen atmosphere. 2-Piperidone (4.2 g, 14 mmol) in dry THF (20 mL) and trimethylsilyl chloride (6.0 mL, 42 mmol) were added to the solution, stirred at −70 °C for 15 min, and warmed to room temperature (solution A). Diisopropylamine (6.0 mL, 45 mmol) and 1.6 M *n*-butyllithium/hexane (30 mL, 48 mmol) were added to dry THF (21 mL) with stirring at −70 °C under a nitrogen atmosphere (solution B). Solution A was added to solution B and stirred at −70 °C after 15 min. Ethyl benzoate (1.75 g, 11.7 mmol) was added to the mixture and stirred for 15 min. The reaction mixture was warmed to room temperature and stirred for 18 h. The resulting precipitate was collected, dissolved in HCl (20 mL), and heated under reflux at 120 °C for 24 h. After cooling, the HCl was removed by an evaporator. The brown residue was recrystallized from methanol/acetone to afford the hydrochloride salt of PTP (611 mg, 33%) as a gray solid: mp 73.8–74.5 °C (dec); ^{1}H NMR δ 1.89 (m, 4-CH_2, 5-CH_2, 4H), 3.28 (br s, 3-CH_2, 2H), 3.75 (br s, 6-CH_2, 2H), 7.65 (t, J = 7.6 Hz, Ph, 2H), 7.77 (t, J = 7.6 Hz, Ph, 1H), 8.00 (d, J = 7.6 Hz, Ph, 2H); HRMS m/z (M^+) calcd for $C_{11}H_{13}N$ 159.1048, found 159.1050.

5-Benzylidene-6-phenyl-2,3,4-trihydropyridine (14). The title compound was obtained from benzaldehyde and PTP, as described for **2**. **14**: a light yellow solid; yield 9.0%; mp 139.1–141.8 °C (dec); ^{1}H NMR δ 1.71 (q, J = 6.1 Hz, 5-CH_2, 2H), 2.77 (dt, J = 1.6, 6.1 Hz, 4-CH_2, 2H), 3.71 (t, J = 6.1 Hz, 6-CH_2, 2H), 6.58 (s, =CH, 1H), 7.28–7.96 (m, Ph, 10H); HRMS m/z (M^+) calcd for $C_{18}H_{17}N$ 247.1361, found 247.1386.

Biology

Preparation of membranes from cockroach nerve cords. The procedure of Orr et al.[26] was modified to prepare the membrane fraction from the ventral nerve cords of cockroaches. Adult male American cockroaches (*P. americana* L.) were dissected in ice-cold 50 mM Tris–HCl buffer containing 200 mM sucrose and 1 mM EDTA (pH 7.4) (buffer A). The nerve cords isolated from 10 cockroaches were homogenized with a glass–Teflon homogenizer (25 strokes) in ice-cold buffer A (2 mL). The homogenate was then centrifuged at 25,000*g* for 30 min. The supernatant was decanted, 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 in the same manner as above. The supernatant was decanted, 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 until use. The protein-dye binding method[27] was used to determine protein concentration, with bovine serum albumin (BSA) as a standard.

Binding assays with [^{3}H]EPI. The procedure of this assay is based generally on the method of Orr et al.[26] Either a 20-μL aliquot of buffer B for the determination of total binding, a 20-μL aliquot of buffer B containing unlabeled (±)-EPI (final concn 5 μM) for the determination of nonspecific 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 [^{3}H]EPI (final concn 2 nM) and buffer B (80 μL) containing nerve cord membranes (40 μg as protein) were added to all test tubes, which were then incubated at 24 °C for 2 h. Whatman GF/C filters were presoaked in buffer B containing 10 mg of BSA/mL for 2 h. The reaction was terminated by rapid filtration through the GF/C filters under reduced pressure, using a Brandel M-24 cell harvester. The filters were rapidly washed twice with 2 mL of 10 °C-cold buffer B containing 2 mg of BSA/mL. The filter disks were removed and placed in 10 mL of toluene–Methyl Cellosolve-based scintillation fluid, and the bound radioactivity was determined using a Beckman LS 6000SE liquid scintillation counter. Test compounds were first dissolved in DMSO and diluted with buffer B. The final concentrations of DMSO in assay solutions were lower than 0.1% (v/v), which did not affect the binding activity of membranes. Each experiment was performed in duplicate and repeated at least three times. IC_{50} values were estimated from the mean values of inhibition percentage at three to five compound concentrations, using the Probit method. The inhibition constant K_i was calculated according to the method of Cheng and Prusoff.[28]

Insecticidal assays. Adult male German cockroaches (*Blattella germanica* L.) were immobilized in an ice-cold jar, and an acetone solution (1 μL) containing PB (30 μg) and NIA16388 (10 μg) was topically applied to each roach abdomen. After being kept at 25 °C for 1 h, the cockroaches were again immobilized in an ice-cold jar. A DMSO solution (0.25 μL) containing a test compound was injected into the ventral side of each roach abdomen. The cockroaches were kept with water at 25 °C, and after 24 h, the mortality rate was recorded. Each dose was given to 10 cockroaches, and each assay was performed in triplicate. LD_{50} values were calculated from the mean values of mortality at five concentrations, using the Probit method.

Molecular modeling and QSAR analysis

The structure of CBA (**1**) was modeled using the molecular modeling software SYBYL, version 6.5.[29] The coordinate was calculated using the SYBYL standard

values for bond lengths and angles. The initial coordinate thus obtained was fully optimized by the semi-empirical molecular orbital method AM1. The pK_i values [log(1/K_i (M))] of 10 *para*-substituted CBA analogues in the inhibition of [^{3}H]EPI binding to *P. americana* nerve cord membranes were analyzed using the QSAR analysis software OREG 2.05.[30]

Acknowledgements

We thank T. Okumoto for his helpful advice.

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