5-Azidoimidacloprid and an Acyclic Analogue as Candidate Photoaffinity Probes for Mammalian and Insect Nicotinic Acetylcholine Receptors

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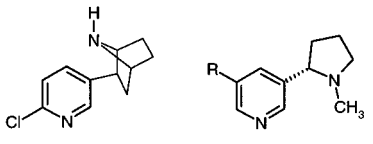
The 5-azido analogue of the major insecticide imidacloprid, 1-(5-azido-6-chloropyridin-3-ylmethyl)-2-nitroiminoimidazolidine (**1**), and an acyclic analogue, *N*-(5-azido-6-chloropyridin-3-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine (**2**), were prepared in good yields as candidate photoaffinity probes for mammalian and insect nicotinic acetylcholine receptors (nAChRs). The essential intermediate was 5-azido-6-chloropyridin-3-ylmethyl chloride (**3**) prepared in two ways: from 6-chloro-5-nitronicotinic acid by selective reduction and then diazotization, and from *N*-(6-chloropyridin-3-ylmethyl)morpholine by an electrophilic azide introduction with lithium diisopropylamide followed by chlorine substitution of morpholine with ethyl chloroformate. Coupling of **3** with 2-nitroiminoimidazolidine gave **1**. Conversion of **3** to **2** was achieved in good yields via the hexahydrotriazine intermediate **14**. Fortuitously, the azido substituent in **1** and **2** increases the affinity 7−79-fold for rat brain and recombinant α4β2 nAChRs (K_is 4.4−60 nM competing with [^{3}H](−)-nicotine) while maintaining high potency on both insect nAChRs (*Drosophila* and *Myzus*) (K_is 1−15 nM competing with [^{3}H]imidacloprid). Azidopyridinyl compounds **1** and **2** are therefore candidate photoaffinity probes for characterization of both mammalian and insect receptors.

Introduction

The nicotinic acetylcholine receptor (nAChR) is the target for a wide variety of candidate and commercial drugs[1] and insecticides.[2] The most potent nAChR agonists contain a 6-chloropyridin-3-yl or a 2-chlorothiazol-5-yl moiety. Important examples are the insecticide imidacloprid and analogues for the insect nAChR[3−5] and the analgesic natural product epibatidine for the mammalian nAChR.[6] Minor structural modifications confer selectivity among the mammalian receptor subtypes[1,7] and between insects and mammals.[8,9]



Photoaffinity probes are important tools in structural analysis of nAChRs. They include [^{3}H]nicotine,[10] [^{3}H]5-azidonicotine,[11] and a 2-azido-5-[^{125}]iodobenzoyloxyethyl derivative of an imidacloprid analogue.[12,13] The latter photoaffinity probe is not ideal because the photoreactive substituent is some distance from the essential binding portion of the molecule. With the information available, the nicotine analogues are selective for mammal nAChRs and the imidacloprid analogues for insect nAChRs.[7,9,14] The preferred photoaffinity ligand would be of nanomolar potency on both mammalian and insect nAChRs so that a single probe could be used for structural analysis in both cases.

Design of the photoaffinity probe candidates was based on known structure−activity relationships of photoactivatable reagents, nAChR agonists, and neonicotinoid insecticides. Aryl azides are the most popular photoaffinity reagents.[15] Preferred neonicotinoid aromatic substituents are chloropyridinyl and chlorothiazolyl,[4,16,17] but only the chloropyridinyl derivatives allow introduction of an azide functionality. The 2(6)-position in the pyridinyl group is not appropriate because 2(6)-azidopyridines are too labile.[18] Two observations focused attention on the 5-azido-6-chloropyridin-3-yl substituent as a suitable candidate for preparation of the photoaffinity probe. First, 5,6-dichloropyridin-3-yl substituted neonicotinoids have excellent insecticidal activity.[19−22] Second, 5-azidonicotine is very potent at the mammalian nAChR.[11]

Two candidate photoaffinity probes were chosen for this study. The first was azidoimidacloprid (**1**) with an imidazolidine moiety. The second was the acyclic analogue (**2**), i.e., the chloropyridinyl counterpart[21,23,24] of the very potent insecticide clothianidin which has a chlorothiazolyl substituent. In each case the 5-azido-6-chloropyridin-3-yl moiety was introduced. Three nAChR targets were considered. Rat nAChR was examined as brain membranes[25] and recombinant α4β2 receptor.[26]

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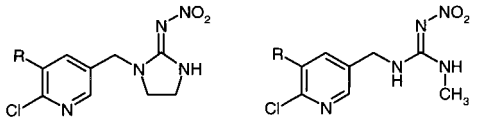


imidacloprid (R=H)
1: azidoimidacloprid (R=N_3)

acyclic analog (R=H)
2: azido acyclic analog (R=N_3)

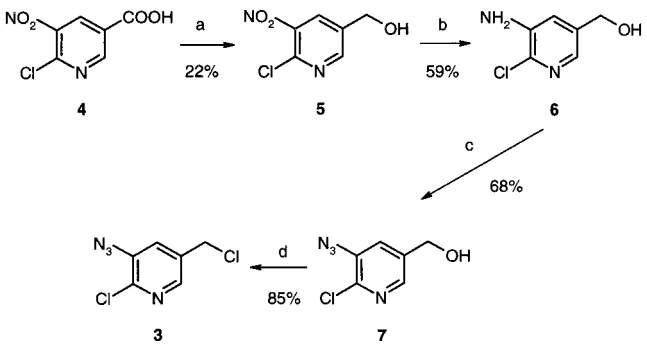
Insect nAChR investigations used *Drosophila melanogaster* brain, where the deduced sequences of the subunits are well established,[9] and *Myzus persicae*, as the target pest insect with major features established for the molecular biology of its nAChR.[27,28]

Results and Discussion

Synthesis of 5-Azido-6-chloropyridin-3-ylmethyl chloride (3). This key intermediate was prepared in two ways. The first method, shown in Scheme 1, started from 6-chloro-5-nitronicotinic acid (**4**),[29] which was selectively reduced at the carboxylic group to alcohol **5** in 22% yield. The borane-dimethyl sulfide complex[30] was better than other reducing reagents tried. The nitro group was reduced to the amine **6**, with retention of the chlorine atom, in 59% yield using activated iron powder in acetic acid.[31] The two-step transformation of the amine **6** to the azide **7** was carried out in good yield. The alcohol **7** was finally converted to the chloromethyl derivative **3** on treatment with $SOCl_2$. The overall yield of 7.5% was less than desirable.

A second synthetic approach was developed to obtain azido derivative **3** (Scheme 2). An azido group can directly be introduced to a phenyl[32] or pyridinyl[33,34] moiety by a metalation reaction using tosyl azide as an electrophile. In π-deficient aza aromatic systems, halogen atoms such as chlorine or fluorine have a strong ortho directing effect.[35−37] Thus, starting from readily accessible 6-chloropyridin-3-ylmethyl chloride (**8**),[38] the chloromethyl group was first protected as the morpholin-4-ylmethyl group (H. Szczepanski, personal communication). The resulting pyridine derivative **9** was lithiated with 2.0 equivalents (equiv) of lithium diisopropylamide (LDA) in tetrahydrofuran (THF) at −78 °C. The anion generated was then cooled to −100 °C and treated as quickly as possible with 2.0 equiv of tosyl azide[39] to afford the azido pyridine **10** in 45% yield. No other isomers could be detected in the crude product, indicating that a clean metalation occurred at the 5-position of the pyridine ring. Freshly prepared LDA and fast quenching of the lithiated intermediate at −100 °C are crucial to obtain good yields. Small variations of these optimized reaction conditions resulted in a clear yield reduction. In one experiment tosyl azide was added at −78 °C over a 5 min period to the lithiated intermediate. After workup and column chromatography, the azido pyridine **10** was obtained only in 22% yield. As a byproduct, the diazido derivative **11** was isolated in 6% yield so a second metalation occurred at the 4-position of the primary product **10**. The diazido pyridine **11** is very unstable. After standing for 16 h at room temperature, the colorless oil had turned black and NMR analysis revealed total decomposition of **11**. Finally, the desired intermediate **3** was obtained in one step from the morpholino derivative **10**. The chloromethyl group in **3** was generated from the morpholinomethyl group in **10** after treatment with ethyl chloroformate. By this new synthetic route the key intermediate **3** was obtained in only three steps from easily available starting materials in an overall yield of 29%.

Scheme 1[a]



4 **5** **6** **3** **7**

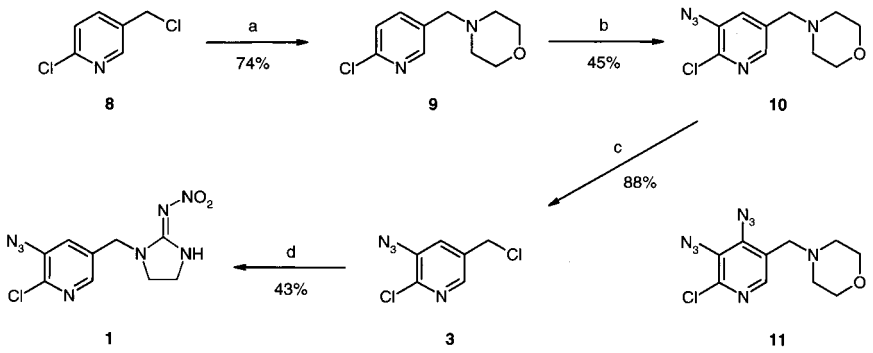
[a] Reagents and conditions: (a) $Me_2S{\cdot}BH_3$, THF; (b) activated Fe, AcOH, 90 °C, 1 min; (c) i. $NaNO_2$, HCl, ii. NH_2OH; (d) $SOCl_2$, $CHCl_3$.

Synthesis of 5-Azidoimidacloprid (1) (Scheme 2). The coupling reaction of **3** with 2-nitroiminoimidazolidine **12**[40] proceeded using K_2CO_3 in acetonitrile to give **1** in 43% yield. Similar yields were obtained if this alkylation reaction was performed in DMF at 50 °C. TLC analysis of the crude material revealed very little formation of dialkylated product. No procedure is currently available for synthesis of [^{3}H]-**1** at high specific activity.

Synthesis of 5-Azido Acyclic Analogue (2). Few useful methods are known for preparation of *N*,*N*′-disubstituted *N*″-nitroguanidines. Alkylation of monosubstituted nitroguanidines with alkylhalides yields complex reaction mixtures.[41] Other approaches starting from *S*-methyl-*N*-nitro-isothiourea are also not satisfactory because only low yields are obtained or many steps are involved.[42,43] However, a suitable method has been reported to achieve the desired product.[41,44] Thus, monosubstituted nitroguanidines are first treated with a primary amine and formalin to afford the corresponding 2-nitroiminohexahydro-1,3,5-triazine derivatives which are converted to *N*,*N*′-disubstituted *N*″-nitroguanidines in high overall yields by alkylation and subsequent acid-catalyzed ring opening (Scheme 3). Following this strategy 6-chloro-5-azidopyridin-3-ylmethyl chloride (**3**) was coupled at 50 °C with 1,5-dimethyl-2-nitroiminohexahydro-1,3,5-triazine (**13**)[22,44] in DMF using K_2CO_3 as a base to give the alkylated product **14** in 83% yield. If the same reaction was performed with NaH as base in a solvent mixture of DMF/THF (1:9), **14** was isolated in only 26% yield. This drastic yield reduction may be due to the instability of 2-nitroiminohexahydro-1,3,5-triazines toward strong basic conditions.[41] Ring cleavage of **14** to the acyclic azido analogue **2** by acidic hydrolysis proceeded in 59% yield.

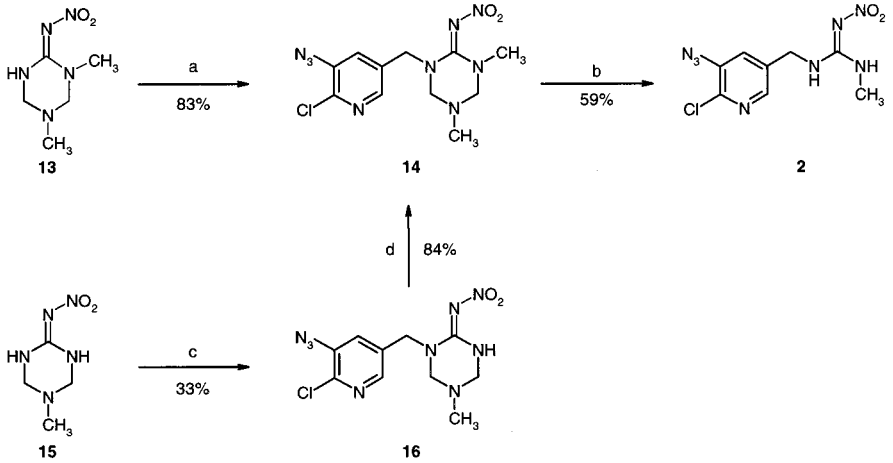
In an alternative route for the synthesis of **14** (Scheme 3), 5-methyl-2-nitroiminohexahydro-1,3,5-triazine **15**[22,44,45] was first alkylated with 5-azido-6-chloropyridin-3-ylmethyl chloride (**3**) in DMF using NaH as a base to give compound **16** in 33% yield. This procedure gave substantial amounts of the dialkylated byproduct detected in the crude material. The final methylation with NaH and 18-crown-6 in DMF followed by methyl iodide gave

Scheme 2[a]



[a] Reagents and conditions: (a) morpholine, toluene; (b) i. LDA, −78 °C; ii. Tos-N_3, −100 °C to 0 °C; (c) $ClCO_2Et$, THF, 60 °C; (d) 2-nitroiminoimidazolidine (**12**), K_2CO_3, MeCN, 70 °C; or K_2CO_3, DMF, 50 °C.

Scheme 3[a]



[a] Reagents and conditions: (a) **3**, K_2CO_3, DMF, 50 °C; (b) 1 N HCl, MeOH; (c) **3**, NaH, DMF; (d) i. NaH, 18-crown-6, MeCN, ii. MeI.

the hexahydrotriazine **14** in 84% yield. This route offers an attractive method to prepare [^{3}H]-**2**.

Photolysis of 1 and 2. Azido derivatives **1** and **2** show absorptions at 260−262 nm and a shoulder at 300 nm characteristic of the aryl azide chromophore[15] and at 268−272 nm corresponding to the nitroguanidine chromophore.[46] The former but not the latter absorptions of both compounds disappear within 2 min on irradiation of **1** or **2** with 300 nm light. Biological studies were made without photoactivation of the azido probes.

Structure−Activity Relationships (Table 1; Figure 1). The effect of the 5-azido substituent was evaluated by affinity comparisons for imidacloprid with **1** and the acyclic analogue with **2** using rat brain, rat α4β2, *Myzus* and *Drosophila* membrane preparations. The parent compounds have relatively poor affinities in rat brain membranes (K_is 290−3500 nM) and the somewhat more sensitive α4β2 preparation (imidacloprid K_i 50 nM), but this is greatly changed with the azido substituent which fortuitously increases the affinity 7−79-fold so that the K_is for **1** and **2** are 44 nM with brain and for **2** is 4.4 nM with α4β2 membranes. All of the compounds have high affinities for the insect preparations (K_is 1−15 nM). The azido substituent increases the affinity with *Myzus* by 1.9−2.7-fold while it decreases the affinity with *Drosophila* by 0.14−0.77-fold. The overall goal of discovering candidate photoaffinity probes of high affinity on both mammalian and insect nAChRs is achieved with azidoimidacloprid (**1**) and its acyclic analogue (**2**). It may therefore be possible to use the same probe for mammals and insects in evaluating the structural basis for selectivity.

Table 1. Effect of 5-Azido Substituent on the Affinity of Imidacloprid and the Acyclic Analogue for Mammalian and Insect nAChRs

receptor, radioligand, and compound	K_i[a] (nM ± SD, n = 3) parent	azido derivative	K_i ratio parent/azido
Mammalian with [^{3}H]Nicotine			
rat brain[b]			
imidacloprid	290 ± 60	44 ± 7	7
acyclic analogue	3500 ± 900	44 ± 8	79
rat α4β2[b]			
imidacloprid	50 ± 10	4.4 ± 1.2	11
Insect with [^{3}H]Imidacloprid			
Myzus			
imidacloprid[b]	1.9 ± 0.5	1.0 ± 0.2	1.9
acyclic analogue	12 ± 2	4.5 ± 0.2	2.7
Drosophila			
imidacloprid	2.1 ± 0.5	15 ± 4	0.14
acyclic analogue	10 ± 2	13 ± 2	0.77

[a] Calculated by the Cheng and Prusoff [47] equation $K_i = IC_{50} \div (1 + [L]/K_d)$ with K_d values of 4.5, 3.3, 2.3, and 2.2 nM for rat brain, rat α4β2,[26] *Myzus*,[5] and *Drosophila*[5] nAChRs, respectively, and radioligand concentrations of 5 nM for [^{3}H]nicotine and 1.5 nM for [^{3}H]imidacloprid. The K_d value for rat brain membranes was calculated using the data for titration of unlabeled (−)-nicotine on the binding of 5 nM [^{3}H]-(−)-nicotine. [b] Inhibition curves are shown in Figure 1.

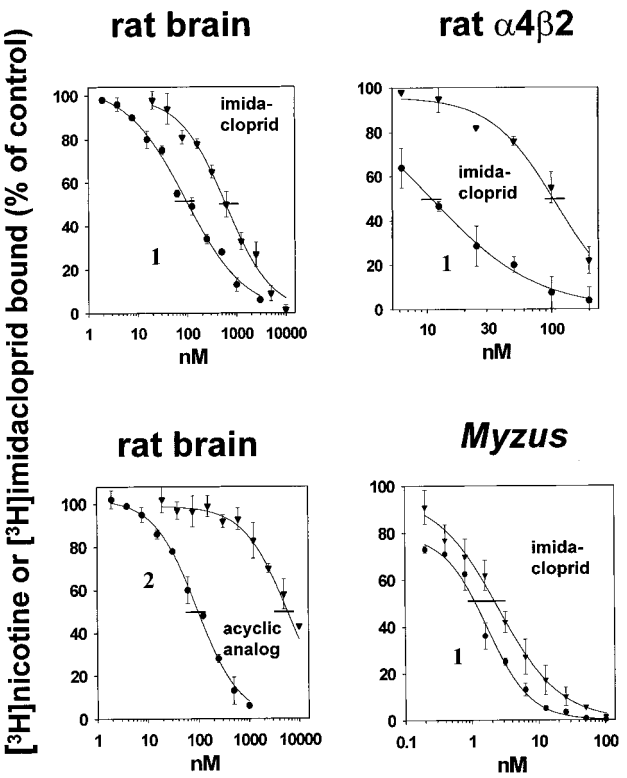


Figure 1. Effect of the 5-azido substituent on the affinity of imidacloprid and the acyclic analogue on [^{3}H]nicotine binding by rat brain and recombinant $\alpha 4\beta 2$ nAChRs and [^{3}H]imidacloprid binding by *Myzus* head membranes.

Experimental Section

Chemical Methods. Silica gel TLC was performed for analysis and determination of *Rf* values with precoated plastic sheets (0.2 mm gel layer) containing fluorescent indicator and for preparative purposes with precoated silica gel GF plates (0.5 mm gel layer). Silica gel 60 for column chromatography was 200−425 mesh. NMR spectra were recorded for solutions in $CDCl_3$, CD_3OD, or DMSO-d_6 using a Bruker AM-300 spectrometer. Chemical shifts are reported in δ (ppm) for ^{1}H at 300 Hz and for ^{13}C at 75 MHz relative to internal tetramethylsilane. Infrared (IR) spectra were recorded in Nujol on a Perkin-Elmer 1600 series Fourier transform IR spectrometer with the bands reported as cm^{-1}. Mass spectra (MS) were obtained using an electron-impact (EI) system (LKB 9000S, direct introduction) or with a fast atom bombardment system (ZAB HF system, VG Analytical Manchester FAB system). Combustion analyses were performed by the Microanalytical Laboratory (College of Chemistry, University of California, Berkeley) or by Novartis AG, Basel, or Solvias AG, Basel, using the prepared compounds without further purification. UV absorbances were measured on a Hewlett-Packard 8452A diode array spectrophotometer. Photoreactivity of the candidate photoaffinity probes was determined for 0.7 mM solutions in absolute ethanol in a 1 cm quartz cell positioned 3 cm from seven 300 nm lamps in a Rayonet photochemical reactor (The Southern New England Ultraviolet Co., Hamden, CT). Melting points are uncorrected. All solvents were of reagent grade. THF was dried by distillation from sodium/benzophenone. Acetonitrile and DMF were dried by storage over molecular sieves.

Each intermediate and candidate probe was >98% pure on the basis of TLC and ^{1}H and ^{13}C NMR integrations. Compounds with photosensitive substitutions were used in subdued light.

6-Chloro-5-nitropyridin-3-ylmethanol (5). To a solution of **4** (202 mg, 1 mmol) in THF (10 mL) was added a solution of borane-dimethyl sulfide complex in THF (0.6 mL, 1.2 mmol) at room temperature under nitrogen atmosphere, and the mixture was stirred for 5 h. The reaction mixture was poured into methanol (10 mL), allowed to stand overnight in a hood, and concentrated on a rotary evaporator. The residual solid was slurried in THF (20 mL), heated to reflux, cooled to 20 °C, filtered, and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column using EtOAc/hexane 1:2 as eluent to give 40 mg of pure product as a pale yellow solid in 22% yield. $Rf = 0.2$ in EtOAc/hexane 1:2. Mp = 58 °C. ^{1}H NMR ($CDCl_3$) δ 8.58 (d, $J = 2.1$ Hz, 1H), 8.28 (d, $J = 2.1$ Hz, 1H), 4.88 (s, 2H, CH_2), 2.80 (bs., 1H, OH). ^{13}C NMR ($CDCl_3$) δ 150.4, 144.7, 142.1, 137.0, 132.5, 60.8. EI-MS (*m*/*z*, rel int.) 190 (M^+, 33%), 188 (M^+, 100%), 159 ($M^+ - CHO$, 51%), 142 ($M^+ - NO_2$, 62%), 112 ($M^+ - CH_2O$, $-NO_2$, 77%). EI-HRMS for $C_6H_5ClN_2O_3$, calcd 187.9988, found 187.9990. Anal. ($C_6H_5ClN_2O_3$) C, H, N.

5-Amino-6-chloropyridin-3-ylmethanol (6). A solution of **5** (320 mg, 1.7 mmol) was heated to 90 °C with activated iron (462 mg, 8.25 mmol) in acetic acid (6 mL) for 1 min. After cooling to room temperature, adding ethanol (20 mL), and filtering from inorganics, the filtrate was concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column using EtOAc/hexane 1:1 as eluent to give 260 mg of pure product as pale yellow crystals in 59% yield. $Rf = 0.5$ in EtOAc. Mp = 85 °C. ^{1}H NMR ($CDCl_3$) δ 7.76 (d, $J = 1.8$ Hz, 1H), 7.10 (d, $J = 1.8$ Hz, 1H), 4.65 (s, 2H, CH_2), 4.11 (s., 2H, NH_2). ^{13}C NMR (DMSO-d_6) δ 140.9, 138.1, 134.6, 133.6, 120.3, 60.1. EI-MS (*m*/*z*, rel int.) 160 (M^+, 32%), 158 (M^+, 100%), 129 ($M^+ - CHO$, 39%), 93 ($M^+ - Cl$, $-CH_2O$, 23%). EI-HRMS for $C_6H_7ClN_2O$, calcd 158.0246, found 158.0248. Anal. ($C_6H_7ClN_2O$) C, H, N.

5-Azido-6-chloropyridin-3-ylmethanol (7). To a mixture of **6** (241 mg, 1.54 mmol), concentrated (37%) HCl (1.4 mL), and water (3.6 mL) in an ice-cold bath was added dropwise a solution of $NaNO_2$ (110 mg, 1.59 mmol) in water (1.1 mL). The mixture was stirred for 15 min at room temperature, and then a solution of $NH_2OH{\cdot}HCl$ (131 mg, 1.87 mmol) in water (1.1 mL) was added. The resulting mixture was poured into a solution of Na_2CO_3 (6.6 g) and water (18 mL) in an ice bath, and this was stirred at 0 °C for 4 h. The mixture was extracted with isopropyl ether (4 × 25 mL), and the combined organic layer was dried over $MgSO_4$ and evaporated. The residue was purified by preparative TLC using EtOAc/hexane 1:1 as eluent to give 190 mg of pure product as colorless crystals in 68% yield. $Rf = 0.8$ in EtOAc/hexane 1:1. Mp = 150 °C (dec). ^{1}H NMR ($CDCl_3$) δ 8.12 (bs, 1H), 7.55 (bs, 1H), 4.76 (d, $J = 5.1$ Hz, 2H, CH_2), 2.07 (t, $J = 5.1$ Hz, OH). ^{13}C NMR ($CDCl_3$) δ 143.4, 136.8, 134.9, 126.4, 124.2, 61.5. IR ν 2121. EI-MS (*m*/*z*, rel int.) 186 (M^+, 20%), 184 (M^+, 61%), 156 ($M^+ - N_2$, 48%), 121 ($M^+ - N_2$, $-Cl$, 60%), 93 (90%), 65 (100%). EI-HRMS for $C_6H_5ClN_4O$, calcd 184.0152, found 184.0149.

5-Azido-6-chloropyridin-3-ylmethyl chloride (3). To a solution of **7** (70 mg, 0.38 mmol) in $CHCl_3$ (5 mL) was added $SOCl_2$ (0.5 mL), and the solution was heated at reflux temperature for 1 h. The reaction mixture was poured into ice−water, extracted with isopropyl ether, dried, and concentrated. The residue was purified by preparative TLC using EtOAc/hexane 1:3 as eluent to give 65 mg of pure product as a yellowish liquid in 85% yield. $Rf = 0.5$ in EtOAc/hexane 1:3. ^{1}H NMR ($CDCl_3$) δ 8.17 (d, $J = 1.8$ Hz, 1H), 7.51 (d, $J = 1.8$ Hz, 1H), 4.58 (s, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ 144.5, 142.7, 135.0, 133.7, 127.6, 41.6. IR ν 2127. EI-MS (*m*/*z*, rel int.) 202 (M^+, 18%), 174 ($M^+ - N_2$, 36%), 167 ($M^+ - Cl$, 60%), 139 ($M^+ - N_2$, $-Cl$, 100%). EI-HRMS for $C_6H_4Cl_2N_4$, calcd 201.9818, found 201.9814. Anal. ($C_6H_4Cl_2N_4$) C, H, N, Cl.

***N*-(6-Chloropyridin-3-ylmethyl)morpholine (9).** A solution of 6-chloropyridin-3-ylmethyl chloride (**8**)[38] (32.6 g, 0.20 mol) and morpholine (38.3 g, 0.44 mol) in toluene (200 mL) was heated at reflux temperature for 8 h. The reaction mixture was poured into brine, extracted with Et_2O, dried with Na_2SO_4, and concentrated under vacuum. The crude was crystallized from hexane to afford 31.9 g (74%) of pure product as colorless crystals. $Rf = 0.31$ in EtOAc. Mp = 65−66 °C. ^{1}H NMR ($CDCl_3$) δ 8.35 (d, $J = 2$ Hz, 1H), 7.69 (dd, $J_1 = 7.5$ Hz,

J_2 = 2 Hz, 1H), 7.31 (d, J = 7.5 Hz, 1H), 3.71 (m, 4H), 3.49 (s, 2H), 2.45 (m, 4H). Anal. ($C_{10}H_{13}ClN_2O$) C, H, N, Cl.

***N*-(5-Azido-6-chloropyridin-3-ylmethyl)morpholine (10).** Method A: A solution of freshly distilled diisopropylamine (15.5 g, 153 mmol) in dry THF (200 mL) was cooled to −30 °C and treated with *n*-butyllithium (91 mL of a 1.6 M solution in hexane, 146 mmol) over a 15 min period. Then the reaction mixture was cooled to −78 °C, and a solution of **9** (15.5 g, 73 mmol) in THF (50 mL) was added dropwise over a period of 10 min. After being stirred for 5 min at −78 °C, the reaction mixture was cooled to −100 °C, and a solution of tosyl azide[40] (28.8 g, 146 mmol) in THF (50 mL) was added within 1 min. During this time the reaction temperature rose to −60 °C. The cooling bath was then removed, and the reaction mixture was allowed to warm to 0 °C over a 45 min period. The mixture was poured into saturated NH_4Cl (200 mL) and extracted with EtOAc. The organic layer was washed with brine, dried with Na_2SO_4, and evaporated. The residue was purified by chromatography on silica gel using EtOAc as eluent to yield 8.3 g (45%) of pure product as slightly yellow oil. Rf = 0.65 in EtOAc. ^{1}H NMR ($CDCl_3$) δ 8.09 (d, J = 2 Hz, 1H), 7.52 (d, J = 2 Hz, 1H), 3.72 (m, 4H), 3.49 (s, 2H), 2.47 (m, 4H). Electrospray-MS (*m*/*z*, rel int.) 254 (MH^+, 50%). EI-HRMS for $C_{10}H_{12}ClN_5O$, calcd 253.0730, found 253.0728. Anal. ($C_{10}H_{12}ClN_5O$) C, H, N, Cl.

Method B: The conditions were varied by using 1/10th of the scale in method A and adding the tosyl azide at −78 °C over a 5 min instead of a 1 min period. Workup as above gave 410 mg (22%) of **10** and 130 mg (6%) of *N*-(4,5-diazido-6-chloropyridin-3-ylmethyl)morpholine (**11**). **11**: Rf = 0.73 in EtOAc. ^{1}H NMR ($CDCl_3$) δ 8.08 (s, 1H), 3.71 (m, 4H), 3.48 (s, 2H), 2.49 (m, 4H). Electrospray-MS (*m*/*z*, rel int.) 295 (MH^+, 100%).

Conversion of 10 to 5-Azido-6-chloropyridin-3-ylmethyl chloride (3). A solution of **10** (7.8 g, 30.7 mmol) in THF (100 mL) was treated with ethyl chloroformate (5.9 mL, 61.5 mmol), then stirred at 60 °C for 6 h and evaporated. The residue was purified by chromatography on silica gel using EtOAc/hexane 1:4 as eluent to yield 5.46 g (88%) of **3**.

1-(5-Azido-6-chloropyridin-3-ylmethyl)-2-nitroiminoimidazolidine (1). Method A: A mixture of **3** (90 mg, 0.39 mmol) and **12** (58 mg, 0.45 mmol) in acetonitrile (10 mL) was stirred with K_2CO_3 (73 mg, 0.54 mmol) at 70 °C for 7 h. After filtration from inorganic salts, which were rinsed with acetonitrile, the filtrate was concentrated, and the residual solid was filtered, rinsed with cold water, and dried. The solid was purified by preparative TLC using EtOAc as eluent to give 50 mg of pure product as colorless crystals in 43% yield. Rf = 0.65 in EtOAc. Mp = 170 °C (dec). ^{1}H NMR ($CDCl_3$) δ 8.19 (bs, 1H, NH), 8.09 (d., J = 1.8 Hz, 1H), 7.51 (d, J = 1.8 Hz, 1H), 4.56 (s, 2H, py-CH_2), 3.82 (m, 2H), 3.54 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 160.3, 144.4, 139.7, 134.0, 132.8, 128.9, 45.1, 44.3, 41.5. IR ν 2127. FAB-LRMS (2-nitrobenzyl alcohol, *m*/*z*) 297 (MH^+), 165, 137. FAB-HRMS for $C_9H_9ClN_8O_2$, calcd 297.0615 (MH^+), found 297.0637. Anal. ($C_9H_9ClN_8O_2$) C, H, N, Cl. UV (EtOH) λmax 262 (log ϵ 4.25), 268 (shoulder, log ϵ 4.20), 300 (shoulder, log ϵ 3.80).

Method B: A mixture of **3** (500 mg, 2.46 mmol), **12** (320 mg, 2.46 mmol), and K_2CO_3 (850 mg, 6.16 mmol) in DMF (10 mL) was stirred at 50 °C for 8 h. After filtration, the filtrate was concentrated and the residue was purified by chromatography on silica gel using EtOAc/MeOH 9:1 as eluent to yield 320 mg (44%) of **1**.

1-(5-Azido-6-chloropyridin-3-ylmethyl)-3,5-dimethyl-2-nitroiminohexahydro-1,3,5-triazine (14). Method A: A mixture of 2-nitroiminohexahydro-1,3,5-triazine (**13**) (1.02 g, 5.9 mmol), **3** (1.20 g, 5.9 mmol), and K_2CO_3 (2.04 g, 14.8 mmol) in DMF (20 mL) was stirred at 50 °C for 18 h. After filtration, the filtrate was concentrated under vacuum, and the residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH 19:1 as eluent to yield 1.66 g (83%) of **14** as colorless crystals. Rf = 0.20 in EtOAc/MeOH 9:1. Mp = 146 °C (dec). ^{1}H NMR ($CDCl_3$) δ 8.08 (d, J = 1.8 Hz, 1H), 7.64 (d, J = 1.8 Hz, 1H), 4.70 (s, 2H, py-CH_2), 4.34 (bs, 2H, CH_2), 4.28 (bs, 2H, CH_2), 3.09 (s, 3H), 2.54 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 157.7, 144.2, 142.7, 135.6, 131.1, 127.9, 70.4, 67.6, 48.6, 40.0, 35.7. IR ν 2123. FAB-LRMS (2-nitrobenzyl alcohol, *m*/*z*) 340 (MH^+), 307, 289. FAB-HRMS for $C_{11}H_{14}ClN_9O_2$, calcd 340.1036 (MH^+), found 340.1041. Anal. ($C_{11}H_{14}ClN_9O_2$) C, H, N, Cl.

Method B: To a suspension of **13** (55 mg, 0.32 mmol) in DMF (0.1 mL) and THF (1.0 mL) was added NaH (15 mg of 60% oil dispersion, 0.38 mmol) at 0 °C. After the mixture was stirred for 20 min, **3** (64 mg, 0.32 mmol) was added, and the mixture was stirred a further 12 h at room temperature and the subsequent 5 h at 50−60 °C. Preparative TLC using EtOAc/MeOH 15:1 as eluent gave 28 mg of pure product **14** as colorless needles from ether in 26% yield.

***N*-(5-Azido-6-chloropyridin-3-ylmethyl)-*N*′-methyl-*N*″-nitroguanidine (2).** To a solution of **14** (500 mg, 1.47 mmol) in MeOH (2 mL) was added 1 N HCl (5 mL), and the resulting reaction mixture was stirred at 80 °C for 90 min. After evaporation, the residue was purified by chromatography on silica gel using EtOAc/MeOH 9:1 as eluent to yield 246 mg (59%) of **2** as colorless crystals. Rf = 0.70 in EtOAc/MeOH 9:1. Mp = 170 °C (dec). ^{1}H NMR (CD_3OD) δ 8.14 (d, J = 2.1 Hz, 1H), 7.78 (d, J = 2.1 Hz, 1H), 4.53 (s, 2H, py-CH_2), 2.95 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 159.6, 145.4, 141.9, 136.3, 136.1, 129.5, 42.5, 28.7. IR ν 2120. UV (EtOH) λmax 260 (log ϵ 4.10), 272 (shoulder, log ϵ 4.05), 300 (shoulder, log ϵ 3.67). FAB-LRMS (glycerol, *m*/*z*) 285 (MH^+). FAB-HRMS for $C_8H_9ClN_8O_2$, calcd 285.0608 (MH^+), found 285.0619. Anal. ($C_8H_9ClN_8O_2$) C, H, N, Cl.

1-(5-Azido-6-chloropyridin-3-ylmethyl)-5-methyl-2-nitroiminohexahydro-1,3,5-triazine (16). To a suspension of NaH (30 mg of 60% oil slurry, 0.75 mmol) in DMF (1 mL) was added 5-methyl-2-nitroiminohexahydro-1,3,5-triazine (**15**) (100 mg, 0.63 mmol) at 0 °C. After the mixture was stirred for 30 min, **3** (100 mg, 0.50 mmol) was added, and the resulting mixture was stirred a further 20 h. NH_4Cl (100 mg) was added to the reaction mixture, and DMF was removed under reduced pressure at below 40 °C. The residue was purified by preparative TLC using EtOAc/$CHCl_3$ 1:9 as eluent to give 66 mg of pure product as colorless crystals in 33% yield. Rf = 0.65 in EtOAc/$CHCl_3$ 1:9. Mp = 156 °C (dec). ^{1}H NMR (DMSO-d_6) δ 9.45 (bs, 1H, NH), 8.20 (d, J = 1.8 Hz, 1H), 7.86 (d, J = 1.8 Hz, 1H), 4.57 (s, 2H, py-CH_2), 4.39 (bs, 2H, CH_2), 4.29 (bs, 2H, CH_2), 2.52 (s, 3H). ^{13}C NMR (DMSO-d_6) δ 154.6, 144.6, 139.5, 134.0, 133.7, 128.8, 67.7, 61.6, 46.0, 38.6. IR ν 2124. FAB-LRMS (2-nitrobenzyl alcohol, *m*/*z*) 326 (MH^+). FAB-HRMS for $C_{10}H_{12}ClN_9O_2$, calcd 326.0880 (MH^+), found 326.0873. Anal. ($C_{10}H_{12}ClN_9O_2$) C, H, N.

Conversion of 16 to 1-(5-Azido-6-chloropyridin-3-ylmethyl)-3,5-dimethyl-2-nitroiminohexahydro-1,3,5-triazine (14). To a suspension of dried oil-free NaH (4.8 mg, 0.2 mmol) in dry acetonitrile (3 mL) with a dried nitrogen atmosphere was added 18-crown-6 (3 mg, 0.01 mmol) and **16** (32.5 mg, 0.1 mmol). After the mixture was stirred at room temperature for 30 min, a solution of dry methyl iodide (14.2 mg, 0.1 mmol) in acetonitrile (0.5 mL) was added, and the resulting mixture was stirred for 1 h. The reaction mixture was subjected without working-up to preparative TLC using EtOAc/MeOH 15:1 as eluent. From the band of Rf 0.2, 28.5 mg of the product was isolated in 84% yield.

Binding Site Assays. Rat Brain Membranes and Recombinant α4β2 nAChR.[25,26] The standard assay involved incubation of 5 nM [^{3}H](−)-nicotine (81 Ci/mmol; NEN Life Science Products, Boston, MA) and various concentrations of inhibitor with 400 μg of protein from rat brain membranes or 100 μg of protein from recombinant rat α4β2 nAChR in 0.25 mL of buffer for 30 min at 22 °C. The binding buffer consisted of 20 mM Tris-HCl, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1.0 mM EDTA. Reactions were terminated by addition of 5 mL of ice-cold 0.9% NaCl followed by rapid vacuum filtration with a cell harvester (Brandel Model MLR-48, Gaithersburg, MD) through GF/B glass fiber filters and two 5 mL washes on the filter. Filters were presoaked at least 60 min in 0.1% w/v polyethylenimine (fresh solution) to reduce nonspecific binding. Specific binding was

defined as total binding with radioligand alone minus nonspecific binding determined with 5 μM (−)-nicotine.

Insect Membranes.[5] For *Myzus* assays, the membrane preparation (200 μg of protein) in membrane isolation buffer (50 μL) [20 mM Na_2HPO_4 (adjusted to pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and protease inhibitors (1 μM chymostatin, 1 μM leupeptin, 1 μM pepstatin, and 100 μM phenylmethanesulfonyl fluoride)] was added to binding buffer (100 μL) [10 mM Na_2HPO_4 (adjusted to pH 7.4) containing 50 mM NaCl] to give a total volume of 200 μL. Binding buffer (25 μL) alone (control) or containing candidate inhibitors was then added. For *Drosophila* assays, the procedure was the same as for *Myzus* with the following changes: 200 μg of protein; membrane isolation buffer consisting of 100 mM Na_2HPO_4 (adjusted to pH 7.4) containing 0.32 M sucrose, 0.1 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, and 100 μM phenylmethanesulfonyl fluoride. In competition studies, unlabeled nicotinoids were assayed with a 2-fold dilution factor, and 10 concentrations centered about the IC_{50} (the concentration for 50% inhibition). The membranes were preincubated with the candidate inhibitor for 5 min before adding [^{3}H]imidacloprid[38] (32 Ci/mmol) in binding buffer (25 μL) at the standard final assay concentration of 1.5 nM (∼50,000 dpm) in 250 μL of reaction mixture. The incubation was for 90 min at 22 °C with shaking. The reaction was terminated by filtering the incubation mixture through GF/B filters presoaked in 0.1% polyethylenimine (fresh solution), followed by two washes each with 5 mL of ice cold washing buffer (10 mM Na_2H PO_4 adjusted to pH 7.4 containing 50 mM NaCl). Nonspecific binding was established with 25 μM unlabeled imidacloprid.

General. Solutions of test compounds were made in binding buffer and ethanol (final concentration <0.05%). All assays were performed with two samples for each concentration and in three independent experiments unless indicated otherwise. The studies were made in subdued light without photoactivation of the azido probes. Compound stability under the specified conditions was verified by TLC. Typical values for specific binding were 2500 dpm for rat brain membranes, 4500 dpm for rat $\alpha4\beta2$ nAChR, 1100 dpm for *Myzus* membranes, and 3000 dpm for *Drosophila* membranes. Specific binding relative to total binding averaged 95%, 99%, 91%, and 80%, respectively. The IC_{50} for each candidate inhibitor was determined by iterative nonlinear least-squares regression using the SigmaPlot program (Jandel Scientific Software, San Rafael, CA). K_i values were derived by the Cheng−Prusoff method.[47]

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Supporting Information Available: Supplementary table giving results of elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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