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Fipronil-based Photoaffinity Probe for *Drosophila* and Human β3 GABA Receptors

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Abstract—Modification of the major insecticide fipronil (**1**) by replacing three pyrazole substituents (hydrogen for both cyano and amino and trifluoromethyldiazirinyl for trifluoromethylsulfinyl) gives a candidate photoaffinity probe (**3**) of high potency (IC_{50} 2–28 nM) in blocking the chloride channel of *Drosophila* and human β3 GABA receptors.

γ-Aminobutyric acid (GABA) receptors are ligand-gated chloride ion channels and the target for important drugs such as benzodiazepines and toxicants including insecticides.[1,2] The GABA-recognition and benzodiazepine binding sites have been labeled and characterized with appropriate photoaffinity probes.[3,4] The most important chloride channel blockers are the botanical toxicant picrotoxinin and three major insecticides, two of which are polychlorocycloalkanes (i.e., α-endosulfan and lindane) and the third is an arylpyrazole (fipronil) (**1**) (Fig. 1).[5–7] The localization of the 'insecticide binding site' (also known as the picrotoxinin or non-competitive blocker site) has only been determined indirectly for lack of a suitable high-affinity photoreactive ligand.[5]

We report here that 1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)diazirinyl]-1*H*-pyrazole (**3**) (Fig. 1) has interesting properties as a candidate photoaffinity probe. This compound was based on **1** and selected for study for two reasons. First, moderate to high receptor potency is retained on deleting the cyano and amino substituents of **1**, as in compound **2** (Fig. 1),[8] and on replacing $-S(O)CF_3$ at the 4-position with $-S(O_2)CF_3$, $-SCF_3$ or $-CF_3$.[9–11] Second, the (trifluoromethyl)diazirinyl moiety, with many structural features in common with this series of substituents, is a favorable carbene generating group for photolabeling reagents.[12]

Introduction of the photoreactive substituent (Scheme 1)[13,14] involved conversion of the bromopyrazole (**4**),[15] via the trifluoromethylketone (**5**),[16] oxime tosylate (**6**),[17] and diaziridine (**7**)[18] to the (trifluoromethyl)diazirine (**3**)[19] in an overall yield of 18%. Compounds were isolated by chromatography on a silica gel column with hexane–ethyl acetate and characterized by ^1H and ^{13}C NMR[20] and HRMS and UV for the end product **3**.[21]

The mammalian $GABA_A$ receptor consists of hetero-oligomeric assemblies of several different subunits (α1-α6, β1-β4, γ1-γ3, and others), which can be studied in various recombinant combinations.[1,2,22–24] There are several lines of evidence that the insecticide binding site is in the lumen of the chloride channel pore at the second transmembrane segment and a β subunit may play an important role. The *Drosophila* GABA receptor has two α and one β subunits (one of which has been cloned and expressed).[25–27] A single point mutation in *Drosophila* greatly reduces the binding affinity for the major GABAergic insecticides, suggesting that this A302S

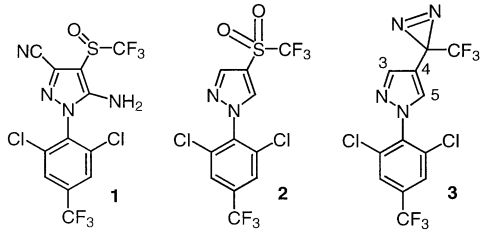

Figure 1. Structures of fipronil (**1**), a potent analogue lacking the amino and cyano substituents (**2**) and the candidate photoaffinity probe (**3**).

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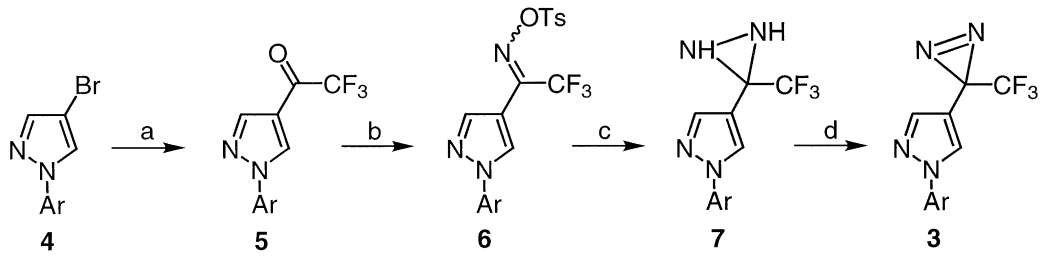

Scheme 1. (a) *n*BuLi, $EtOC(O)CF_3$, THF, −78 °C, 40%; (b) (1) $NH_2OH{\cdot}HCl$, pyridine; (2) TsCl, pyridine, 64%; (c) NH_3, Et_2O, 72%; (d) Ag_2O, Et_2O 97%.

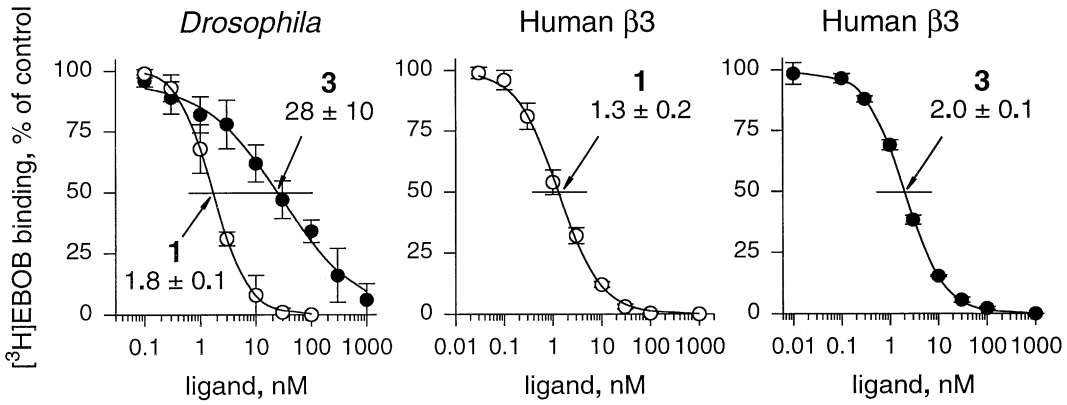

Figure 2. Potencies of fipronil (**1**) and the candidate photoaffinity probe (**3**) as inhibitors of [^{3}H]EBOB binding in *Drosophila* and human β3 GABA receptors. IC_{50} values (as an index of potency for the insecticide binding site of GABA receptors) are given as nM ± SD ($n = 3$). Data points include SD. Hill coefficients (n_H) for **1** with the *Drosophila* and human β3 receptors and for **3** with the β3 receptor are 1.01 ± 0.07, 1.02 ± 0.02 and 1.06 ± 0.04, respectively, suggesting a similar type of action. Interestingly, a n_H of 0.61 ± 0.02 is obtained for **3** with *Drosophila* receptor, implying a multiple class of action or negative cooperativity.

modification is at or near the insecticide binding site.[25,26,28] This region of the *Drosophila* GABA receptor has a close sequence homology to that of the human β3 subunit.[22,24,25] The mammalian β3 homooligomeric receptor displays binding activity of radiolabeled blocker(s) appropriate for the insecticide binding site when expressed in either insect Sf9 cells[23,24] or human embryonic kidney cells.[29,30] The human recombinant β3 receptor from Sf9 cells is more sensitive to insecticide action than a receptor with any other single subunit or combination of subunits examined.[23] Not only the insecticide sensitivity but also the specificity are conserved, so it is proposed that the insecticide and/or picrotoxinin binding site of the human β3 homooligomer can be used as a model for the *Drosophila* receptor and vice versa.[24] A single photoaffinity probe for both the *Drosophila* and human GABA receptors would allow a more direct test of this hypothesis. It would also help define the precise location of the insecticide binding site in insect and mammalian GABA receptors and potentially the basis for selective toxicity.

The insecticide binding site can be readily examined with 4′-ethynyl-4-[2,3-$^3H_2$]propylbicycloorthobenzoate ([^{3}H]EBOB), which has similar affinity at *Drosophila* head and human β3 homooligomeric receptors.[23,24,28,31] The potencies of **1** and **3** in displacing specific [^{3}H]EBOB binding were therefore compared with *Drosophila* and human β3 receptors[32] with the fortuitous finding that high potency is retained for **3** on both receptors (Fig. 2). The molar concentrations for 50% inhibition (IC_{50} values) by **1** and **3** with both receptors fall in the 1–28 nM range. The structural changes on modifying **1** to give **3** apparently have little effect on fit at the receptor, with a potency loss of only 1.5- to 16-fold, that is candidate photoaffinity probe **3** is a suitable mimic of **1** at the binding site in the GABA receptors of both insects and mammals. The final step in converting the candidate photoaffinity probe into a practical photoaffinity radioligand will require the incorporation of one or two tritium atoms, either at the phenyl substituent or the position of newly introduced protons on converting **1** to **3**.

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15. Bromopyrazole **4** was prepared by bromination of the corresponding unsubstituted pyrazole with bromine in glacial acetic acid.
16. Bromopyrazole **4** (1.264 g, 3.51 mmol) in dry THF (12 mL) at −78 °C was added slowly to *n*-butyllithium (1.6 M, 2.6 mL, 4.16 mmol) in dry THF (20 mL) at −78 °C. After stirring for 15 min, $EtOC(O)CF_3$ (1.0 g, 7.0 mmol) was added in THF (5 mL). The solution was allowed to warm to 20 °C and stirred for 1 h and 1 mL of saturated NaCl was added. The reaction mixture was diluted with ethyl acetate (20 mL) and washed with half-saturated NaCl (50 mL) and saturated NaCl (50 mL) then the organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was chromatographed on silica gel with 5% ethyl acetate in hexane to obtain **5** (529 mg, 1.40 mmol, 40%).
17. Trifluoromethylketone derivative **5** (529 mg, 1.40 mmol) was dissolved in pyridine (4.4 mL, dried over CaH_2) and ethanol (1.8 mL). $NH_2OH{\cdot}HCl$ (100 mg, 1.44 mmol) was added and the mixture was stirred overnight at 60 °C. The residue from evaporation was dissolved in ethyl acetate with work up involving washing with water, 0.1 N HCl and saturated NaCl, then drying, filtration and concentration. The crude oxime intermediate (401 mg, 1.02 mmol) was dissolved in anhydrous pyridine (3.5 mL) at room temperature, tosyl chloride (278 mg, 1.46 mmol) was added and the mixture was refluxed for 4 h. The solvent was evaporated and the residue chromatographed with 4–6% ethyl acetate in hexane to give **6** (490 mg, 0.90 mmol, overall 64%) as a white solid.
18. A solution of **6** (390 mg, 0.714 mmol) in dry ether (1.5 mL) in a thick-walled glass tube was cooled to −78 °C and ~0.5 mL NH_3 was condensed into it. The screw cap was closed tightly and the mixture was stirred overnight at room temperature. The residue after removing the white precipitate was chromatographed with 15% ethyl acetate in hexane to obtain **7** (oil, 202 mg, 0.516 mmol, 72%) which is stable for several months at −20 °C.
19. To **7** (202 mg, 0.516 mmol) in dry ether (4 mL) was added freshly prepared[14] Ag_2O (500 mg, 2.16 mmol). After 45 min, the mixture was filtered, concentrated, and chromatographed (3% ethyl acetate in hexane) to obtain **3** (oil, 194 mg, 0.50 mmol, 97%). Compound **3** is much less stable than immediate precursor **7**, so the final conversion step should be carried out shortly before use.
20. ^1H NMR ($CDCl_3$, 300 MHz, δ) for pyrazole H_3 and H_5, respectively: **3** 7.68 and 7.55; **4** 7.80 and 7.61; **5** 8.38 and 8.30; **6** 8.44 and 8.26; **7** 7.96 and 7.82. ^{13}C NMR ($CDCl_3$, 75 MHz, δ) for CF_3 and pyrazole C_3, C_4 and C_5, respectively: **3**, 121.8, 139.8, 113.5, and 126.0; **4**, no CF_3, 142.4, 95.4, and 125.9; **5** 116.3, 143.0, 117.9, and 126.2; **6** 119.9, 146.3, 108.2 and 126.0; **7** 123.3, 140.4, 115.8, and 126.6.
21. **3:** HRMS (EI) *m/z* $C_{12}H_5Cl_2F_6N_4$, $[MH]^+$ calcd 388.9796, found 388.9798; UV (ethanol) μ_{max} 358 nm.
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32. The receptor preparations were *Drosophila* head membranes (300 μg protein/assay) and Sf9 cell membranes expressing recombinant human β3 receptor (250 μg protein/assay). Assay mixtures contained membrane fraction, various concentrations of **1** or **3** dissolved in dimethyl sulfoxide (final concentration 1%), and 0.8 nM (*Drosophila*) or 1.0 nM (β3) [^3H]EBOB (30 Ci/mmol) in a total volume of 0.5 mL of 300 mM (for *Drosophila*) or 200 mM (for β3) sodium chloride/10 mM sodium phosphate buffer (pH 7.5). After 70 min incubation at 25 °C, the binding reaction was terminated by filtration on a GF/B filter (presoaked in 0.1% polyethyleneimine) and the filter was rinsed three times with ice cold water containing 0.9% sodium chloride/2% ethanol. Specific binding was defined as the difference in radioactivity on the filter in the absence and presence of 5 μM α-endosulfan.