Original Article

Synthesis of a Photoreactive and Fluorescent Pyrethroid and Its Nerve Effects

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A photoreactive and fluorescent ester, named "KUP-1," was synthesized as a possible probe for studies at the primary site of action of pyrethroids. Nerve effects of the compound were fundamentally the same as those induced by conventional pyrethroids. KUP-1 induced the depolarizing afterpotential in the falling phase of the action potential and depolarized the resting membranes in crayfish and cockroach giant axons. The compound slowed the decay of the sodium currents measured in the crayfish giant axon under the voltage-clamp conditions. The maximum wavelength of the emission spectrum was apart enough to distinguish the compound from proteins, indicating that the compound could be used as a tool for photo-affinity labeling.

INTRODUCTION

Pyrethroids are widely used for insect control not only in domestic spaces but also in agriculture. Their neurotoxic effects play a key role in insect toxicity.^{1,2)} These insecticides induce repetitive discharges and/or conduction blocks in excised nerve preparations. Electrophysiological studies using voltage-clamp techniques have revealed that these insecticides selectively inhibit the closing of the voltage-sensitive sodium channels, thereby altering the normal conduction of nerve impulses.³⁻⁵⁾

The ²²Na⁺ uptake of the neuroblastoma cells or synaptosomal fractions from rat brains has been measured, showing that pyrethroids themselves did not enhance the sodium influx, but enhanced the flow induced by veratridine and some other neurotoxins.⁶⁻⁸⁾ Binding experiments have examined the interaction of pyrethroids with membrane preparations from house fly heads⁹⁾ or purified and reconstituted rat-brain sodium channels.¹⁰⁾ The interac-

tion was monitored only as the allosteric enhancement of the specific binding of tritiated batrachotoxinin A 20 α -benzoate.^{9,10)} However, no evidence to show the direct binding of the radiolabeled pyrethroids to the sodium channels has been presented.

The phenotypes of pyrethroid-resistant tobacco budworms¹¹⁾ and house flies¹²⁾ due to their nerve insensitivity are tightly linked with changes in the chromosomal locus encoding the sodium channels. These findings support the notion that the sodium channel is the most important site involved in the nerve effects of pyrethroids. Nevertheless, genetic analyses do not necessarily show the direct interaction of these compounds with the channel proteins.

To understand the exact binding site of pyrethroids, we designed a compound having a photoreactive azide group as well as a fluorescent chromophore (Fig. 1). We showed that this compound, KUP-1, exerted nerve effects similar to those of conventional pyrethroids.

Fig. 1 Chemical structure of KUP-1.

MATERIALS AND METHODS

1. General Methods

The ^1H NMR spectra of compounds were recorded by JEOL JNM 270 spectrometer (270 MHz) with TMS as an internal standard. The mass spectra (MS) were recorded by Shimadzu 9100-MK and high-resolution measurements of millimass units of the molecular ion peak were performed on JEOL HX-110 spectrometer with an electron impact (EI) of 70 eV. IR spectra of the compounds were measured using a Shimadzu FTIR-8200D spectrometer, and UV and fluorescence spectra of KUP-1 were measured by Shimadzu UV-240 and RF-5000 spectrometers, respectively. Reagent-grade chemicals were used to synthesize compounds without further purification. Wakogel C-300 (Wako Pure Chemical Industries) and silicagel 60 F₂₅₄ (Merck) were used for column and thin layer chromatographies, respectively. Melting points were not corrected.

2. Synthesis of KUP-1

2.1 2-(4-Azidophenyl)-3-methylbutanoic acid

The acid moiety of KUP-1, 2-(4-azidophenyl)-3-methylbutanoic acid, was synthesized from 4-nitrophenylacetonitrile as described by Adams.¹³⁾ In brief, the starting material was reacted with 2-iodopropane in the presence of potassium methoxide in DMSO at room temperature to give 2-(4-nitrophenyl)-3-methylbutyronitrile. The nitrile was hydrolyzed by 50% sulfuric acid under reflux to give 2-(4-nitrophenyl)-3-methylbutanoic acid. The carboxylic acid was esterified with benzyl alcohol. The nitro compound was reduced by iron powder in acetic acid, diazotized by sodium nitrite and treated with sodium azide. The acid moiety of KUP-1 was finally obtained by alkaline hydrolysis of the ester. mp: 98–100°C (lit. 95–97°C). EI-MS m/z (relative intensity, %): obsd. 219.1008 (M^+ , 13),

219.1009 calcd. for $\text{C}_{11}\text{H}_{13}\text{O}_2\text{N}_3$: 191 ($\text{M}^+ - \text{N}_2$, 30), 176 ($\text{M}^+ - i\text{-propyl}$, 17), 149 (100), 131 (20), 104 (30). ^1H NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$ ppm: 0.68 (3H, d, $J=6.5$ Hz, $-\text{CH}(\text{CH}_3)_2$), 1.06 (3H, d, $J=6.5$ Hz, $-\text{CH}(\text{CH}_3)_2$), 2.17–2.37 (1H, m, $-\text{CH}(\text{CH}_3)_2$), 3.11 (1H, d, $J=10.0$ Hz, $\text{Ar}-\text{CH}(\text{COOH})-$), 7.02 (2H, d, $J=8.5$ Hz, $\text{Ar}-\text{H}$), 7.37 (2H, d, $J=8.5$ Hz, $\text{Ar}-\text{H}$).

2.2 4-Methoxyphthalimide

4-Methoxyphthalimide was synthesized as described.¹⁴⁾ One gram of 4-methoxyphthalic acid (5.1 mmol) and 620 mg of urea (10.3 mmol) were dissolved in 10 ml of ethylene glycol and stirred at 180°C for 5 min. After cooling the reaction mixture to room temperature, yellowish precipitates were collected by filtration, washed with water and dried. The solids were recrystallized from glacial acetic acid, giving colorless needles (790 mg, 88% yield). mp: 221–223°C. EI-MS m/z (relative intensity, %): obsd. 177.0420 (M^+ , 100), 177.0426 calcd. for $\text{C}_9\text{H}_7\text{O}_3\text{N}$: 134 (55), 106 (67). ^1H NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$ ppm: 3.94 (3H, s, phthalimide 4- OCH_3), 7.31 (1H, dd, $J=2.5$, 8.5 Hz, phthalimide C5-H), 7.41 (1H, d, $J=2.5$ Hz, phthalimide C3-H), 7.81 (1H, d, $J=8.5$ Hz, phthalimide C6-H).

2.3 N-Hydroxymethyl-4-methoxyphthalimide

This compound was synthesized as described for *N*-hydroxymethylphthalimide.¹⁵⁾ Five hundred milligrams of 4-methoxyphthalimide (2.82 mmol) were suspended in 1.4 ml of 10.6% formalin and stirred at 105°C for 1 hr. After cooling the solution to room temperature, white precipitates were collected by filtration, washed with water and dried. Recrystallization of the solids from benzene yielded colorless needles (382 mg, 65% yield). mp: 141–143°C. EI-MS m/z (relative intensity, %): obsd. 207.0529 (M^+ , 0.5), 207.0532 calcd. for $\text{C}_{10}\text{H}_9\text{O}_4\text{N}$: 177 (100), 134 (67), 106 (75). ^1H NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$ ppm: 3.95 (3H, s, $\text{Ar}-\text{OCH}_3$), 5.10 (2H, s, $\text{N}-\text{CH}_2\text{OH}$), 7.31 (1H, dd, $J=2.5$, 8.0 Hz, phthalimide C5-H), 7.41 (1H, d, $J=2.5$ Hz, phthalimide C3-H), 7.81 (1H, d, $J=8.0$ Hz, phthalimide C6-H).

2.4 4-Methoxyphthalimide-*N*-methyl 2-(4-azidophenyl)-3-methylbutanoate (KUP-1)

One hundred milligrams of 2-(4-azido-phenyl)-3-methylbutanoic acid (456 nmol) were dissolved in 5 ml of thionyl chloride and reflux-

ed for 2 hr. The solution was evaporated and the residual acid chloride was dissolved in 3 ml of dichloromethane, which was added dropwise to 2 ml of pyridine solution containing 50 mg of *N*-hydroxymethyl-4-methoxy-phthalimide (241 nmol) at 0°C, then stirred at room temperature for 2 hr. The solution was diluted in water and extracted with ether. The combined ethereal extract was washed successively with 2 N HCl, water, saturated NaHCO₃, and saturated brine, and then dried over anhydrous MgSO₄. After evaporating the solvent, the oily residue was purified by silicagel column chromatography with *n*-hexane-ethyl acetate (3:1) as an eluent to yield 88 mg of a slightly yellowish oil, which was solidified by storage in a freezer overnight. Yield: 89%. mp: 84–85°C. UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (ϵ): 238 (45,170), 320 (3380). EI-MS m/z (relative intensity, %): obsd. 408.1442 (M^+ , 0.2), 408.1435 calcd. for C₂₁H₂₀O₃N₄: 380 ($M^+ - N_2$, 0.8), 190 (100). IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 2127 (N₃), 1784 (C=O, phthalimide), 1734 (C=O, ester and phthalimide). ¹H NMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$ ppm: 0.69 (3H, d, $J=6.5$ Hz, $-\text{CH}(\text{CH}_3)_2$), 1.02 (3H, d, $J=6.5$ Hz, $-\text{CH}(\text{CH}_3)_2$), 2.20–2.37 (1H, m, $-\text{CH}(\text{CH}_3)_2$), 3.13 (1H, d, $J=10.0$ Hz, Ar-CH(CO)-CH-(CH₃)₂), 5.63 (1H, d, $J=10.5$ Hz, $-\text{OCH}_2\text{-N}$), 5.72 (1H, d, $J=10.5$ Hz, $-\text{OCH}_2\text{-N}$), 6.92 (2H, d, $J=8.5$ Hz, acid moiety Ar-H), 7.22 (1H, dd, $J=2.5, 8.5$ Hz, phthalimide C5-H), 7.27 (2H, d, $J=8.5$ Hz, acid moiety Ar-H), 7.36 (1H, d, $J=2.5$ Hz, phthalimide C3-H), 7.81 (1H, d, $J=8.5$ Hz, phthalimide C6-H). Fluorescence $\text{Em}_{\text{max}}^{\text{EtOH}}$ nm (Ex. mm): 425 (329), relative quantum yield: 0.06 (ref. quinine sulfate in 0.5 M H₂SO₄ at 20°C).

3. Measurement of Membrane Potentials

Desheathed circumoesophageal connectives of male and female of crayfishes (*Procambarus clarkii*) and intact central nerve cords excised from male adult American cockroaches (*Periplaneta americana*) were used as nerve preparations. The crayfish tissues were immersed in modified van Harreveld solution and the membrane potential of the medial giant axon was recorded intracellularly through a glass capillary electrode filled with 2 M potassium acetate.¹⁶⁾ The resistance of the electrode was 4–8 M Ω . Membrane potentials of

the cockroach giant axons between the 4th and 5th abdominal ganglia were similarly measured, except that the saline was replaced with Ringer's solution and the resistance of the electrode was between 10–20 M Ω .¹⁷⁾ In both axonal preparations, action potentials were generated by electrical stimulation (2–7 V, 0.1 msec duration) with a suction electrode.

Test solutions were prepared by dissolving a small methanol solution of the compound in the salines, then applied by external perfusion. The final concentration of methanol was 1% or less (v/v). Methanol at this concentration range had no effect on the membrane potential. Experiments were performed at 23 $\pm$ 1°C.

4. Measurement of Membrane Currents

Membrane currents were measured in crayfish medial giant axons under voltage-clamp conditions using the sucrose gap method.^{18–21)} The inward sodium current was measured after eliminating the outward potassium current by internal perfusion of the axon with saline containing cesium, instead of potassium ions.^{18–21)} The holding membrane potential was –100 mV during the studies. The ohmic leakage and capacitive currents associated with step changes in the membrane potential were estimated²⁰⁾ and subtracted from the observed currents. The current data were stored and analyzed as described.²⁰⁾ Membrane currents were smoothed as described by Savitzky & Golay.²²⁾ The compound was applied to the axon by internal perfusion of the test solution, which was prepared by adding a small amount of a DMSO solution of the compound to the internal solution. The final concentration of DMSO was 0.5% or lower (v/v). DMSO at this concentration range did not affect the membrane currents. Measurements were taken at 6 $\pm$ 1°C.

RESULTS AND DISCUSSION

KUP-1 elevated the falling phase of the action potential to give a depolarizing afterpotential (Fig. 2A₂). The effect was similar in the cockroach giant axon (Fig. 2B₂). Exposing the cockroach axonal membrane to 1.0 $\times$ 10⁻⁵ M KUP-1 increased the maximum height of the depolarizing afterpotential, leading to repetitive firing of the membrane evoked by a

single stimulus (data not shown).

The resting membranes of both crayfish and cockroach giant axons were depolarized by KUP-1 at concentrations above 1.0×10^{-5} M. An example of the effect on the crayfish axonal membrane is shown in Fig. 3. After

Fig. 2 Action potentials generated in the crayfish (A_1 and A_2) and cockroach (B_1 and B_2) giant axons.

A_1 and B_1 are controls for A_2 and B_2 , respectively. A_2 , 16 min after the application of 2.0×10^{-6} M KUP-1; B_2 , 20 min after the application of 1.0×10^{-6} M KUP-1. Horizontal lines indicate the zero potential level. Arrows in A_2 and B_2 indicate the depolarizing afterpotential caused by the compound.

starting treatment, the resting potential gradually increased with time and sometimes, the membrane potential oscillated for a few minutes. The membrane potential completely recovered from the depolarization upon adding 1.0×10^{-5} M tetrodotoxin, which is a selective blocker of the ion flux through the sodium channels (Fig. 3), demonstrating that the depolarization is attributable to an increase in the sodium permeability of the sodium channels. This type of change in the membrane potential is caused by some pyrethroids.²³⁻²⁵⁾

The inward membrane current was measured by means of a step depolarization of the mem-

Fig. 3 Effects of KUP-1 and tetrodotoxin (TTX) on the resting membrane potential of the crayfish giant axon.

The nerve preparation was treated with 2.0×10^{-5} M KUP-1 at time zero and TTX (1.0×10^{-5} M) was added at 40 min. The arrow indicates oscillation of the membrane potential.

Fig. 4 Effects of KUP-1 and kadethrin on the sodium currents recorded from crayfish giant axons in response to step depolarization from the holding potential (-100 mV) to -20 mV.

In A, a is the control and b is the record at 28 min after internal application of 2.0×10^{-5} M KUP-1. In B, a is control and b is the record at 34 min after application of 2.0×10^{-5} M kadethrin. Arrows 1 and 2 in each graph indicate residual and tail currents, respectively. The dotted lines indicate the zero current level.

brane (-20 mV) from the holding potential (-100 mV), under the voltage-clamp conditions. The inward current rapidly increased with step depolarization of the membrane, and then decayed to the zero level at the end of the depolarizing pulse. This current was due to the inward movement of sodium ions.²⁶⁾ KUP-1 markedly slowed the decay of the sodium current to give the residual current (Fig. 4Ab, arrow 1). Upon step repolarization of the membrane to the holding potential, the compound prolonged the tail current (Fig. 4Ab, arrow 2). These effects are essentially the same as those observed for kadethrin (Fig. 4Bb), suggesting that KUP-1 modulated the sodium channels to remain open.

From these nerve effects, KUP-1 is expected to have insecticidal effects. In fact, the compound showed the lethal effect against American cockroaches (*P. americana*) by the injection method.²⁷⁾ The log (1/MLD) value, where MLD is the minimum lethal dose in mol to kill the cockroaches in the presence of oxidative and hydrolytic metabolic inhibitors,²⁷⁾ was 7.88. Thus, the compound is insecticidally less active than kadethrin [$\log (1/\text{MLD}) = 9.41^{28)}$] by a factor of about 30 and than tetramethrin [$\log (1/\text{MLD}) = 8.42^{27)}$] by a factor of only three.

Having a photochemically-reactive azide moiety, KUP-1 may be more specifically reactive with the site of interaction than non-photoreactive probes. Even though the fluorescence intensity of KUP-1 was not very strong, the maximum wavelength of the emission spectrum was far enough from that of proteins. Because KUP-1 is a non-radioactive compound, it can be handled easily. Thus, the compound would be a useful tool for monitoring labeled proteins.

In conclusion, KUP-1, containing a photoactivable azide group as well as a fluorescent chromophore, induced neurotoxic effects like conventional pyrethroids. Therefore, this compound probably shares the same target site as pyrethroids in nerve preparations and may be useful for photoaffinity labeling.

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要 約

蛍光発色団を有する光反応性ピレスロイドの合成とその神経生理活性

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神経作用性殺虫剤であるピレスロイドの作用点を解明するために、蛍光発色団を有する光反応性化合物、KUP-1、を合成した。KUP-1の蛍光はタンパク質の蛍光より長波長側に極大値を示した。アメリカザリガニおよびワモンゴキブリの神経軸索標本を用いて膜電位に対する効果を測定したところ、KUP-1はピレスロイドと同様に脱分極性後電位を誘起し、静止膜を脱分極させた。また、化合物はアメリカザリガニの神経軸索標本で測定したナトリウムイオン電流に対してもピレスロイド様の電流変化を引き起こした。これらの結果から、KUP-1はピレスロイドの標的タンパク質を同定するための光反応性プローブとして用いることができるものと考えた。