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Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor

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Summary. Studies of extracts of *Delphinium* seeds, long known to be insecticidal, revealed that a principal insecticidal toxin was methyllycaconitine, which is shown to be a potent inhibitor of α-bungarotoxin binding to housefly heads ($K_{inh} = 2.5 \times 10^{-10} \pm 0.5 \times 10^{-10}$M).
Key words. Insecticide; nicotinic receptor; methyllycaconitine; *Delphinium* alkaloids.

The extract of seeds from *Delphinium* has a long history of being used as an insecticide. Pliny the Elder described the use of this seed extract as a topical treatment for 'vermin in the head and other parts of the body'[2].

Recently, work on toxic components of *Delphinium brownii*, a cattle-stock poison in Western Canada, has led to the identification of a principal toxic component, methyllycaconitine (MLA, fig. 1) which displays neuromuscular blocking activity at nicotinic receptors in a rat phrenic nerve-diaphragm preparation[3]. We report here the results of a study on the insecticidal activity of the seeds of *Delphinium* hybrid, cv. 'Pacific Giant, King Arthur' and the identification of a possible site of action at the insect nicotinic receptor.

Chloroform extracts of crushed seeds of the *Delphinium* plant were tested for insecticidal activity against a number of species of insects and mites (table) and found to produce mortality and protect leaves from feeding damage. This extract was also tested for nicotinic activity against insect cholinergic receptors in an assay measuring the inhibition of [^{3}H]-propionyl-α-bungarotoxin (^{3}H α-BGTx) binding to *Musca domestica* head homogenate[4]. α-Bungarotoxin is an antagonist of nicotinic acetylcholine receptors in insects[5], having a $K_D = 1.1 \pm 0.1$ nM. The *Delphinium* seed extract displayed a very potent inhibition of binding of the radioligand to the receptor, being much more potent than the standard, nicotine. In an effort to identify this cholinergic agent, extracts of *Delphinium* seeds were separated into alkaloidal and non-alkaloidal fractions. Both the cholinergic and insecticidal activities were found to reside in the alkaloidal fraction. The alkaloidal fraction was further separated by preparative silica gel thin layer chromatography (TLC) yielding 6 clearly defined alkaloidal fractions. One of these, the major alkaloid, with an R_f of 0.43 on silica gel (Merck, Silica Gel 60, 0.25 mm) when eluted with cyclohexane:chloroform:diethylamine (5:4:1) was found to have very potent cholinergic activity, having a K_{inh} value for displacing ^{3}H α-BGTx of less than 0.5 nM. This fraction was chemically characterized by mass spectrometry, proton and C^{13} NMR spectroscopy and identified as methyllycaconitine. This MLA fraction was also tested and found to display good insecticidal activity against *Spodoptera eridania* and *Musca domestica* (data not shown). A sample of MLA.citrate was obtained and tested for activity in the insect nicotinic cholinergic receptor assay. The MLA.citrate displayed identical cholinergic activity to that observed for the MLA alkaloid fraction purified from *Delphinium* seeds by TLC (fig. 2). The K_{inh} value calculated for MLA.citrate was $2.5 \times 10^{-10} \pm 0.5 \times 10^{-10}$ M. The cholinergic activity of this alkaloid at the insect nicotinic receptor is much more potent than that reported for the rat muscle receptor, where the ED_{50} was determined to be 2.3×10^{-6} M[3]. Aconitine, another aconite alkaloid reported to be more potent than MLA at the rat muscle nicotinic receptor, was found to be considerably less active than MLA at the insect receptor, having a K_{inh} of $2.7 \times 10^{-4} \pm 0.8 \times 10^{-4}$ M. Lycoctonine, which lacks the aromatic ester function of MLA (fig. 1), also inhibited 3H α-BGTx binding to the insect cholinergic receptor, but with a K_{inh} of $3.8 \times 10^{-7} \pm 0.6 \times 10^{-7}$ M. In addition to its lower potency at inhibiting ^{3}H α-BGTx binding, it was also ineffective as an insecticide when tested against *Spodoptera eridania*.

The rank order of potency for inhibition of α-bungarotoxin binding in the insect preparation is MLA > lycoctonine > aconitine; in the rat phrenic nerve-diaphragm preparation[3]

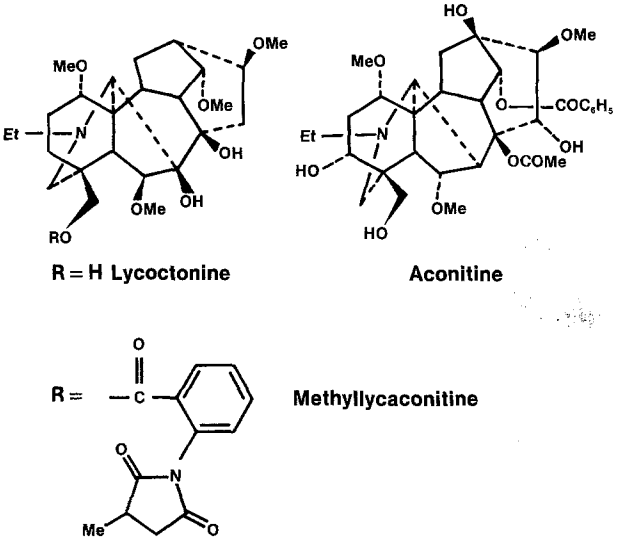

Figure 1. Structures of aconite alkaloids investigated.

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the rank order of potency of these alkaloids is different, being aconitine > MLA > lycoctonine.

MLA.citrate was tested for insecticidal and acaricidal activity (table and fig. 3) and found to have pesticidal action against those insects for which the original *Delphinium* seed extract was effective (*Tetranychus urticae* and *Anopheles quadrimaculatus* are thought to be killed by saponins in the seed extract and are thus not affected by either the insecticidal alkaloid fraction or MLA). This similarity in the insecticidal properties of the seed extract and MLA suggests that the major insecticidal agent in *Delphinium* is MLA and furthermore, that its mode of action may be associated with its high affinity for the insect nicotinic cholinergic receptor. The activity seen with the three alkaloids MLA, lycoctonine and aconitine (fig. 2) is clearly different from that reported for the rat diaphragm muscle nicotinic receptor, and indicates a significant difference in pharmacology of the binding site between mammalian muscle and insect nerve tissue.

Methyllycaconitine provided significant protection of bean leaves against feeding damage by *Spodoptera* larvae at concentrations as low as 100 ppm. Feeding damage was slight and observed mortality was rapid, significant kill being observed in less than 24 h, and surviving larvae did not increase in size. The rapid kill and significant leaf protection seen at 24 h is shown in figure 3. In this particular experiment, feeding damage to the leaf was less than 5% after 72 h (N = 4), whereas controls exhibited feeding damage greater than 95% (N = 4) after the same interval of time. A dose response study of MLA on *Spodoptera* was carried out by exposing sets of 10, third instar larvae to lima bean leaves dipped into a solution of MLA in 2:1 acetone/water. The LC_{50} calculated was 308 ± 48 ppm (SE). Nicotine in the same study had an estimated LC_{50} much greater than 1000 ppm. It may be that MLA has evolved in the plant as a highly specific ligand for the insect nicotinic receptor and acts as an effective antifeedant/natural insecticide by its action on this very important target site in the insect. Methyllycaconitine has a broader spectrum of insecticidal activity than that seen for the cholinergic agonist, nicotine; MLA displays insecticidal activity against *Lepidopteran* insect pests, an activity not seen with nicotine. The Kinh for nicotine, calculated from the results of 6 separate experiments is 8.2 ± 0.6 µM. Nicotine is thus over 10,000-fold less active as an inhibitor of 3H α-BGTx binding to *Musca domestica* head homogenates and this difference in potency may contribute to the differences seen in insecticidal action.

The research on mammalian muscle and plate with MLA[6] suggests that this alkaloid acts as an antagonist. Preliminary experiments (A. Chalmers, unpublished) suggest that MLA, unlike nicotine, does not display agonist activity at the cholinergic cercal nerve synapse in *Periplaneta americana* but does elicit synaptic block. This is consistent with MLA acting as a cholinergic antagonist.

The inhibition reported for MLA in the fly head nicotinic receptor assay is the most potent ever reported for a non-proteinaceous toxin at this site in insects. The very high potency (K_{inh} = 0.25 nM) of this alkaloid for inhibiting the binding of ^{3}Hα-BGT to housefly head tissue (being more potent than the snake toxin itself) suggests that it may be useful as a neurobiological tool for the study of the comparative pharmacology of invertebrate nicotinic acetylcholine receptors.

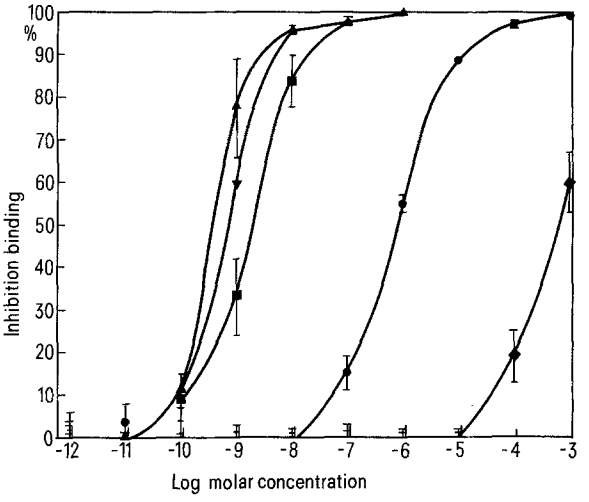

Figure 2. Inhibitory effect of test compound on binding of ^{3}H α-BGTx to *Musca domestica* head homogenates. Tissue homogenates were prepared as described[4]. Test compounds were preincubated for 20 min with the tissue homogenate at 22–24 °C in pH 7.4 sodium phosphate buffer containing 1 mg/ml bovine serum albumin and 9% DMSO. The binding reaction was carried out for 30 min with 5 nM ^{3}H α-BGTx, and terminated by filtration on Whatman GF/C Filters. Data shown are percentage inhibition of binding ± SD. ▲, MLA.citrate; ▼, MLA purified from *Delphinium* seed; ■, α-BGTx; ●, lycocotonine; ◆, aconitine.

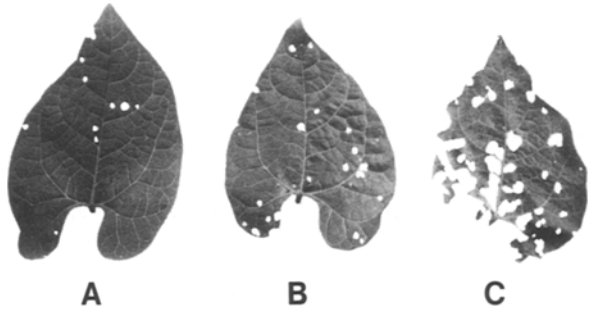

Figure 3. The insecticidal effect of methyllycaconitine against *Spodoptera eridania*. Limabean leaves were dipped in 2:1 acetone:water solutions containing 1000 ppm MLA.citrate (A), 300 ppm MLA.citrate (B), acetone:water control (C). Leaves were exposed to 5, 3rd instar *Spodoptera eridania* larvae for 24 h at an ambient temperature of 27 °C.

Insecticidal acaricidal activity of *Delphinium* extracts compared with MLA

		Chloroform extract	Alkaloid fraction	MLA.citrate
Spodoptera eridania	Larvae	+	+	+
Aphis fabae		−	N.D.	−
Tetranychus urticae		+	−	−
Diabrotica undecimpunctata howardi	Larvae	−	N.D.	−
Anopheles quadrimaculatus	Adult	+	N.D.	−
	Larvae	N.D.	−	−
	Eggs	+	−	−
Empoasca abrupta		+	+	+
Heliothis virescens	Larvae	+	+	+
	Eggs	+	+	+
Musca domestica		+	N.D.	+

(+) active, (−) inactive, (N.D.) not determined. Activity on eggs indicates a contact ovicidal activity, reducing viable egghatch. Active denotes significant (50% +) mortality at a screening rate of 1000 ppm (*Spodoptera, Heliothis* larvae, *Musca*). 300 ppm (*Heliothis* eggs) or 100 ppm (*Empoasca*).

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Renal effects of the inhibitor of thromboxane A_2-synthetase OKY-046[1]

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Summary. Acute renal failure (ARF) was associated with increased urinary thromboxane (TXA_2) excretion and lessened excretion of sodium ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}%). The inhibitor of thromboxane A_2-synthetase OKY-046 enhanced sodium excretion and fractional excretion of sodium in normal and saline loaded animals whereas it partially prevented the reduction in sodium excretion and creatinine clearance and significantly increased fractional excretion of sodium in glycerol treated rats suggesting a partial protection against the development of acute renal failure.
Key words. OKY-046; thromboxane A_2; prostaglandin E_2; prostaglandin I_2; sodium excretion; volume expansion; acute renal failure.

The role of prostaglandins in sodium excretion and in the development of ARF has been widely investigated, since the prostaglandin system has been found to be involved in the regulation of renal hemodynamics[3]. In particular, it was observed that both infusion and increased biosynthesis of vasodilator prostaglandins, PGE_1, PGE_2 and PGI_2 cause enhanced sodium excretion by increasing renal hemodynamics and by inhibiting the tubular reabsorption of sodium[4-11].

In the case of glycerol-induced ARF, it has been shown that, in its early phase, ARF is accompanied by increased renal vascular resistance and diminished renal plasma flow, glomerular filtration rate and sodium excretion[12-15]. The mechanism responsible for these hemodynamic changes is not yet clearly established. the role of catecholamines has been found to be disputable[14,16], and that of the renin-angiotensin system remains controversial[17]. On the other hand, the prostaglandin system seems to be implicated in the early phase of ARF, as is shown by the following findings: a) chronic use of prostaglandin-synthesis inhibitors leads to the development of ARF[18,19], b) PGE_1, PGE_2 and PGI_2 infusions protect rats against ARF[13,20-23] and c) TXA_2 biosynthesis was found to increase during glycerol-induced ARF[24,25].

However, the role of TXA_2 in sodium excretion or in ARF has not been investigated. TXA_2 is a vasoconstrictor[26] and platelet aggregating agent[27], and thus it has physiological action opposite to PGE_1, PGE_2 and PGI_2. It was thus plausible that TXA_2 also plays an opposite role in the case of sodium excretion and ARF development. In order to examine this hypothesis, we used a newly synthetized selective inhibitor of TXA_2-synthetase, OKY-046 (Ono and Kissei Pharm. Co Osaka, Japan)[28].

Our results suggest that TXA_2 has an antinatriuretic action and is implicated in the early phase of glycerol-induced ARF.

Material and methods. All studies were carried out with female Wistar rats weighing 220–235 g. Tap water and standard rat chow were available ad libitum till the day of the experiment. The room temperature at which the animals were maintained was between 22–25 °C and humidity was 35–40%. The animals were randomly allocated to 9 groups; each group contained 9 rats.

Group 1 (Normal Rats) (NR). One hour before the beginning of the experiment the animals were injected i.p. with 1 ml/kg isotonic saline.

Group 2 (NR+OKY-046 2.5 mg/kg). One hour before the beginning of the experiment the animals were injected i.p. with 2.5 mg (10 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Group 3 (NR+OKY-046 25 mg/kg). One hour before the beginning of the experiment the animals were injected i.p. with 25 mg (100 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Group 4 (Volume expanded rats) (VE). One hour before volume expansion with 75 ml/kg isotonic saline s.c., the animals received i.p. 1 ml/kg isotonic saline.

Group 5 (VE+OKY-046 2.5 mg/kg). One hour before volume expansion as in group 4, the animals received i.p. 2.5 mg (10 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Group 6 (VE+OKY-046 25 mg/kg). One hour before volume expansion as in group 4, the animals received i.p. 25 mg (100 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Group 7 (Glycerol treated animals). One hour before the s.c. injection of 10 ml/kg, 50% v/v glycerol in isotonic saline, the animals received i.p. 1 ml/kg isotonic saline.

Group 8 (Glycerol+OKY-046 2.5 mg/kg). One hour before the s.c. injection of glycerol as in group 7, the animals received i.p. 2.5 mg (10 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Group 9 (Glycerol+OKY-046 25 mg/kg). One hour before the s.c. injection of glycerol as in group 7, the animals received i.p. 25 mg (100 µmol)/kg OKY-046 dissolved in 1 ml isotonic saline.

Intraperitoneal injections were carried out one hour before subcutaneous injections, in order to allow OKY-046 to exercise its inhibitory action. Two different doses of the inhibitor were used. In all cases, 6-h urine collections were made, using individual metabolic cages. The animals then were anesthetized and 3 ml of blood were collected from a femoral artery.

The following parameters were measured: 1) Urinary and plasma creatinine concentrations by a method using Fuller's earth in order to eliminate chromogens. 2) Urinary and plasma sodium concentrations by flame photometry. 3) Urinary thromboxane B_2 (TXB_2), 6ketoprostaglandin $F_{1\alpha}$ (6ketoPGF$_{1\alpha}$) (the stable metabolites of TXA_2 and PGI_2 respectively) and PGE_2 by radioimmunoassay.

Clearance of creatinine (C_{Cr}), sodium excretion rate ($U_{Na}V$), and fractional excretion of sodium (FE_{Na}%) were calculated as usual. C_{Cr} was utilized to represent glomerular filtration rate (GFR).