# STUDIES ON THE MODE OF ACTION OF SPINOSAD, THE ACTIVE INGREDIENT IN TRACER® INSECT CONTROL

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## Abstract

Studies defining the mode of action of spinosad are summarized, using the American cockroach as an experimental insect. Spinosad is a naturally occurring mixture of two closely related macrocyclic lactones, known as spinosyns, produced by the actinomycete *Saccharopolyspora spinosa*. *In vivo* studies showed that spinosyns caused widespread excitation of neurons in the central nervous system, leading to involuntary muscle contractions and tremors. At a threshold dose, spinosyn A was estimated by radiotracer measurements to reach an internal equivalent aqueous concentration of approximately 20 nM, and this concentration was sufficient to directly excite the isolated cockroach central nervous system. Furthermore, in isolated neurons, the excitation was found to be due to persistent activation of nicotinic acetylcholine receptors and prolongation of acetylcholine responses by a novel mechanism that distinguishes spinosad from all other nicotinic agonists. Under certain conditions, spinosyns also had effects on γ-aminobutyric acid receptors, but their contribution to symptoms has not been established. Because of its novel mode of action, spinosad has an excellent resistance management profile; with no known cross-resistance, it can be rotated with all other classes of existing and experimental products. Nevertheless, DowElanco is recommending a strong, pro-active resistance management program for Tracer® in cotton.

## Introduction

Spinosad is the active ingredient in a new natural insect control product that will be introduced by DowElanco in 1997 for control of lepidopterous pests in cotton, under the trade name of Tracer (Trademark of DowElanco). Spinosad is a naturally occurring mixture of two active components, 85 % spinosyn A and 15 % spinosyn D (Figure 1), produced by the soil actinomycete *Saccharopolyspora spinosa* (Kirst *et al.*, 1992). Structurally, these compounds are macrolides, based on a unique tetracyclic ring system to which two different sugars are attached. The sugar on the right in Figure 1 is 2,3,4-tri-*o*-methylrhamnose, and that on the left is forosamine. The core tetracyclic ring system contains a unique 12-membered macrocyclic lactone ring. Biological activity of the spinosyns has been published (Sparks *et al.*, 1995; Sparks *et al*., 1996, De Amicis *et al.*, 1997). The activity of spinosyns A and D is similar, so spinosyn A was used in mode of action studies.

Spinosad has a number of outstanding attributes that make it an exciting new tool for Integrated Pest Management (IPM) and Insecticide Resistance Management (IRM), and many of these, including its high efficacy on target insects, safety to beneficial insects, low mammalian and nontarget toxicity, and especially its lack of known cross-resistance problems, are related to its unique mode of action.

This paper gives a brief overview of work carried out at DowElanco to define the mode of action of spinosyns. It is shown that these novel insect control materials activate nicotinic receptors in insect neurons by a novel mechanism, and that this effect can account qualitatively and quantitatively for the nervous system excitation that underlies poisoning symptoms. These findings will be published in a series of more extensive papers in the near future.

## Results and Discussion

### The Mode of Action is Novel

An attempt is often made to determine the target site of a novel insect control material by testing it in assays for known insecticidal target sites. This approach was tried with spinosad at DowElanco against the target sites shown in Table 1, but a mode of action was not identified. In addition, spinosad was tested by external laboratories in more than 60 assays for various drug and toxin target sites, with no significant effects. These negative results suggested that the target site was novel.

With no clues from biochemical assays as to the mode of action, a systematic approach, beginning with symptoms, was taken to identify the target site. Spinosyn A was used as the model compound, and American cockroaches were used as the test insects, because they are amenable to many types of experimental measurements.

### Spinosyns Cause Involuntary Movements

Spinosyns are excitatory, but do not cause the hyperactivity and convulsions that are typical of many traditional insecticides. One of the first symptoms shown by cockroaches injected with spinosyn A was an elevation of the body, caused by extension and depression of the legs (Figure 2). Interestingly, these symptoms even persisted after the insect was decapitated (Figure 2D). Eventually, the legs extended so much that the insect toppled over. Insects also showed other symptoms of excitation, such as wing beating and abdominal bloating, caused by swallowing large amounts of air.

Similar symptoms of abnormal muscle contractions were also shown by other insects, including fruit flies, tobacco budworm larvae and house flies. Tobacco budworm larvae exposed to spinosyns typically fall on their backs and

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exhibit widespread tremors. The true legs were extended, and trembled, as did the mouthparts and even the surface of the cuticle. These excitatory effects were not obvious to the naked eye, but were easily seen with a stereomicroscope. Eventually the movements ceased and the insects were paralyzed.

The difference in symptoms between spinosad and most traditional insecticides should be kept in mind when scouting cotton fields sprayed with Tracer. Whereas traditional insecticides cause worms to convulse and fall off of the leaves quickly, spinosad-treated insects, though incapacitated, remain on the leaves longer.

**Spinosyns Excite the CNS *in vivo* and *in vitro***

In order to determine the gross effects of spinosyns on the neuromuscular system that caused these muscle contractions and tremors, electrophysiological techniques were used to observe nerve and muscle activity in treated insects that were exhibiting symptoms. The diagram in Figure 3 illustrates these experiments with an insect segment containing a leg and a nerve ganglion, which is part of the central nervous system (CNS). The ganglion contains nerve cells or neurons, some of whose normal function is to provide motor control of leg muscles. The neurons have cell bodies in the ganglion, from which emanate long processes. Some of these processes connect to other neurons in the ganglion, while others, the motor axons, exit the ganglion through nerves, which carry them to the muscles. In insects with spinosyn-induced muscle contractions, direct recordings from leg muscles of the treated insects (oscilloscope traces on left) show constant firing of action potentials, indicating that the muscles are being activated by excessive nerve activity. Recordings from the motor nerves also show greatly increased activity (traces in lower right), which can be shown to come from the central nervous system.

Next, it was estimated from radiotracer measurements that spinosyn A must attain an internal aqueous concentration near 20 nM in order to produce symptoms. This concentration applied in a saline bath was just sufficient to stimulate activity in isolated cockroach ganglia (Figure 4). In other words, spinosyn A achieves a concentration in treated insects that is sufficient to cause the observed symptoms by direct actions on nerve ganglia.

**CNS Excitation is Due to Depolarization of Central Neurons**

When microelectrodes were used to record from single neuron cell bodies within isolated ganglia, spinosyn A was found to depolarize the cells and cause them to fire action potentials at a high rate (upper right pair of oscilloscope traces). Note the decrease in the resting potential on the millivolt scale and the heavily clipped action potentials in the presence of spinosyn A.

This additional information shows that spinosyn A exerts its excitatory effects in ganglia by depolarizing the neurons within them, leading to increased firing rates. Increased firing of motor neurons leads to muscle contractions and tremors. At this point, we are at the threshold of understanding the mode of action of spinosyns -- the key is to find the mechanism by which they depolarize neurons.

**Neuronal Depolarization is Due to Activation of Nicotinic Receptors by a Novel Mechanism**

The mechanism by which spinosyn A depolarizes neurons was investigated in studies on neuron cell bodies isolated from the cockroach central nervous system (Figure 5). These neurons, which adhere to the bottom of a petri dish, were impaled with a sharp microelectrode and voltage clamped with a single-electrode voltage-clamp circuit, allowing currents across the membrane to be measured. The neurotransmitter acetylcholine was pulsed onto the neurons from a nearby pipette. The central graph shows a response to ACh in the absence (smooth trace) and presence (dashed trace) of 30 nM spinosyn A. Spinosyn A induced an inward baseline current shift of 0.7 nA and prolonged the ACh response. The effect of spinosyn A on baseline or holding current is shown more clearly on a slow time scale in the graph on the right. Thirty nM had a profound effect. The graph on the left shows that the selective nicotinic antagonist α-bungarotoxin blocked the spinosyn A-induced holding current (open symbols) with the same concentration dependence as it blocked the peak ACh response (filled symbols).

These results show that spinosyn A activates nicotinic receptors and that this effect can account for the neuronal depolarization and resultant excitation of the central nervous system. Furthermore, the prolongation of the ACh response by spinosyn A shows that spinosyn A and ACh act on the receptors simultaneously and therefore at distinct target sites. This conclusion is also supported by the observation that 1 μM spinosyn A did not compete with, or reduce the potency of, α-bungarotoxin in its block of the spinosyn-induced current. These properties are unique among nicotinic agonists, and indicate that spinosyn A activates the receptors by a novel mechanism that is independent of the conventional agonist site.

**Nicotinic Potency is Correlated with Tobacco Budworm Activity**

In addition to the close association seen between nicotinic effects, nerve excitation and symptoms, it is important to show a good correlation between *in vivo* and *in vitro* activity, especially against target pests, so in Figure 6 is shown the correlation between *in vitro* activity against cockroach nicotinic receptors and *in vivo* tobacco budworm biological activity, from an insect screen. Both activities are described by indices, for which higher numbers mean lower activity, and vice versa. The slope of the best-fit line is 0.91, which is close to the theoretical value of 1 expected for a causative relation, and $r^2$ is 0.72, indicating that the

cockroach nicotinic activity can explain 72 % of the variation in TBW activity. This is excellent, considering the difference in species and the expected precision of screening data.

**Insecticide Modes of Action in the Nervous System**

The modes of action of insect control agents that are selective for the nervous system are illustrated in Figure 7. Depiction of a target site on a protein is meant to show that there is a site through which the compound exerts its effect. While there is good evidence that some of the compounds bind directly to the proteins, this has not yet been shown for others, including spinosyns, and it is possible that the effect is mediated by a modulatory protein. The spinosyn box on the nicotinic receptor indicates that this is known to be an important target site, based on the evidence presented above.

Spinosyns are also shown in this diagram to affect GABA receptors. This has been observed in some cells under certain conditions, but we have not been able to show that effects on GABA receptors are important for the insecticidal activity of spinosyns. The effects of spinosyns on GABA receptors are similar in some ways to the effects of avermectins on GABA receptors, but the primary target site for avermectins is thought to be a glutamate receptor (Duce *et al.*, 1995). The sites where both these groups of compounds act on the GABA receptor (which may be a single common site) are shown by ellipses, indicating the tenuous link to insect activity.

**Spinosyns Suffer No Known Cross-Resistance**

The mechanisms of action of a new pest control product is of great interest from the standpoint of resistance management. History has shown and continues to provide new examples, that resistance to any pest control agent will eventually occur. The three main mechanisms of resistance are reduced penetration, increased metabolism and target site insensitivity. Target site insensitivity has arisen more than once in all three of the widely-exploited insecticide target sites: acetylcholinesterase, the voltage-dependent sodium channel and the GABA receptor-activated chloride channel. There is also evidence in house flies of a low level of target site insensitivity to avermectins (Konno and Scott, 1991). This is indicated by the shading of these sites in Figure 7. Resistance management programs attempt to delay the development of resistance by rotation of insecticides with different chemistries and modes of action. Thus, the addition of the novel mode of action of spinosad to the insect control armamentarium permits better resistance management. This conclusion is further supported by studies conducted at DowElanco and elsewhere, using insect strains containing a variety of resistance mechanisms, which have shown that spinosad activity is not affected by any known target site resistance mechanisms. These studies have included strains with target site resistance to pyrethroids, cyclodienes, organophosphates and avermectin (J.G. Scott, Unpublished Observations). In addition, four strains of diamondback moth resistant to various insecticides by various metabolic mechanisms did not show significant cross-resistance to spinosyn A (C.-N. Sun, cited in Sparks *et al*., 1995).

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**References**

De Amicis, C. V., J. E. Dripps, C. J. Hatton and L. L. Karr, 1997, The physical and biological properties of the spinosyns, novel macrolide pest control agents from fermentation, in Phytochemical Pest Control Agents (P.A. Hedin and R.M. Hollingworth, eds.) Amer. Chem. Soc., Wash., D.C., in press.

DowElanco, Spinosad Technical Guide, 1996.

Duce, I.R., N.S. Bhandal, R.H. Scott and T.M. Norris, 1995, Effects of Ivermectin on γ-aminobutyric acid and glutamate-gated chloride conductance in arthropod skeletal muscle, in Molecular Action of Insecticides on Ion Channels (Clark, J.M., ed.) Amer. Chem. Soc., Washington, D.C. pp. 251-263.

Kirst, H. A., K. H. Michel, J. S. Mynderse, E. H. Chao, R. C. Yao, W. M. Nakatsukasa, L. D. Boeck, J. Occlowitz, J. W. Paschel, J. B. Deeter and G. D. Thompson, 1992, Discovery, isolation and structure elucidation of a family of structurally unique fermentation-derived tetracyclic macrolides, in Synthesis and Chemistry of Agrochemicals III (D. R. Baker, J. G. Fenyes and J. J. Steffens, eds.), Am. Chem. Soc., Washington, D. C. pp 214-225.

Konno, Y. and J.G. Scott, 1991, Biochemistry and genetics of abamectin resistance in the house fly, Pestic. Biochem. Physiol. 41, 21-28.

Sparks, T.C., H. A. Kirst, J. S. Mynderse, G.D. Thompson, J.R. Turner, O.K. Jantz, M.B. Hertlein, L.L. Larson, P.J. Baker, M.C. Broughton, J.D. Busacca, L.C. Creemer, M.L. Huber, J.W. Martin, W.M. Nakatsukasa, J.W. Paschal and T.V. Worden, 1996, Chemistry and biology of the spinosyns: components of spinosad (Tracer®), the first entry into DowElanco's naturalyte class of insect control products, Proceedings Beltwide Cotton Conferences, 692-696.

Sparks, T.C., G.D. Thompson, L.L. Larson, H.A. Kirst, O.K. Jantz, T.V. Worden, M.B. Hertlein and J.D. Busacca, 1995, Biological characteristics of the spinosyns: a new naturally derived insect control agents (sic.), Proceedings Beltwide Cotton Conferences, 903-907.

Table 1. Assays for known insecticide target sites could not identify the mode of action of spinosad. Compiled from results of the authors and C.P. Chang, R. Gajewski, N. Orr and A. Shaffner.

| Example Target | Insecticides | Assay type |
|---|---|---|
| electron transport | fenazaqin | electron trnsprt. |
| AchE | OP, carbamate | enzyme assay |
| avermectin site | avermectin, emamectin | binding, physiol. |
| GABA receptor | fipronil, cyclodienes | binding, physiol. |
| sodium channel | DDT, pyrethroids | binding, physiol. |
| nicotinic receptor | imidacloprid, nicotine | binding |
| octopamine receptor | amitraz, chlordimeform | binding, cAMP |

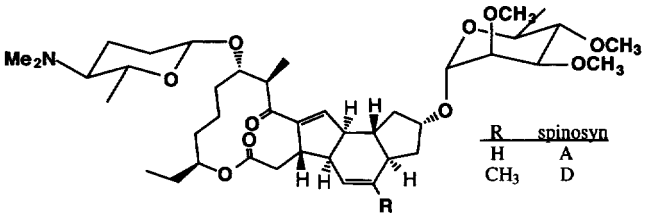


Figure 1. Spinosad, the active ingredient in Tracer® insect control, is a naturally occurring mixture of primarily two active components, 85 % spinosyn A and 15 % spinosyn D, produced by the actinomycete *Saccharopolyspora spinosa*.

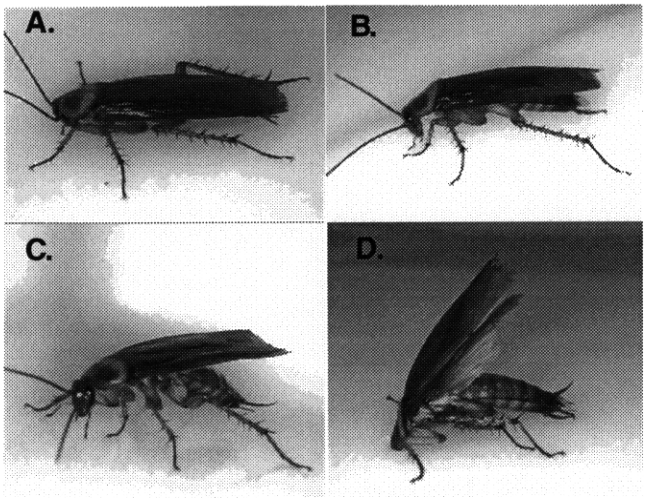


Figure 2. Typical symptoms shown by American cockroaches after injection with spinosyn A, in this case at 2 µg. A. Control. B., C. Depression of the legs elevates the body. Note how all joints are extended. D. The symptoms persist even after decapitation, and even worsen, as the whole body flexes.

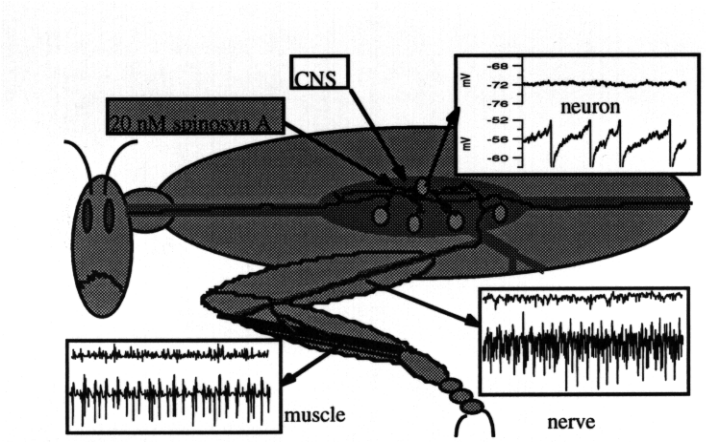


Figure 3. Spinosyns depolarize insect neurons, causing excitation. A diagrammatic insect segment is shown, with pairs of oscilloscope recordings from various locations before (upper trace) and during (lower trace) exposure to spinosyn A. Spinosyn A attains an estimated internal aqueous concentration of 20 nM at a threshold dose. Neurons in the central nervous system (upper right trace pair) are depolarized and excited by spinosyn A (effect of 1 µM spinosyn A is shown, although lower concentrations were also effective). Excitation beginning in the central neurons spreads via nerves (lower right) to muscles (lower left).

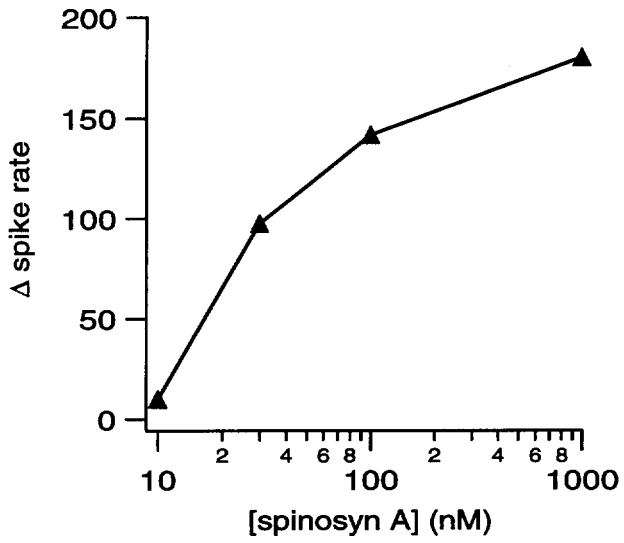


Figure 4. Increase in spike rate in nerves of isolated cockroach ganglia exposed to spinosyn A. The concentration that caused a half-maximal increase was 32 nM.

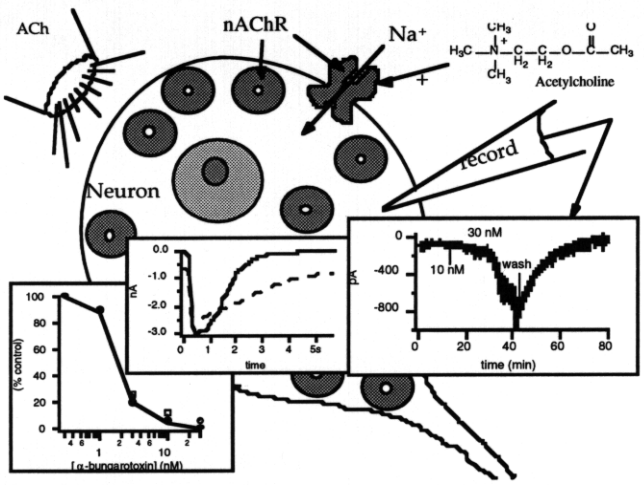


Figure 5. In isolated neuron cell bodies studied with the single-electrode voltage clamp technique, ACh, pulsed from a nearby pipette, activated nicotinic receptors on the cell surface, allowing $Na^+$ ions to flow in. Spinosyns also activated these same receptors. The graph on the right shows the increase of holding current by 10 and 30 nM spinosyn A on a very slow time scale. The center graph compares ACh responses at an expanded time scale in the absence (solid trace) and presence (dashed trace) of 30 nM spinosyn A. The response was greatly prolonged by spinosyn A. The graph on the left shows the concentration dependence of block of the peak ACh response (filled circles) and of the 1 μM spinosyn A-induced holding current (open symbols) by α-bungarotoxin.

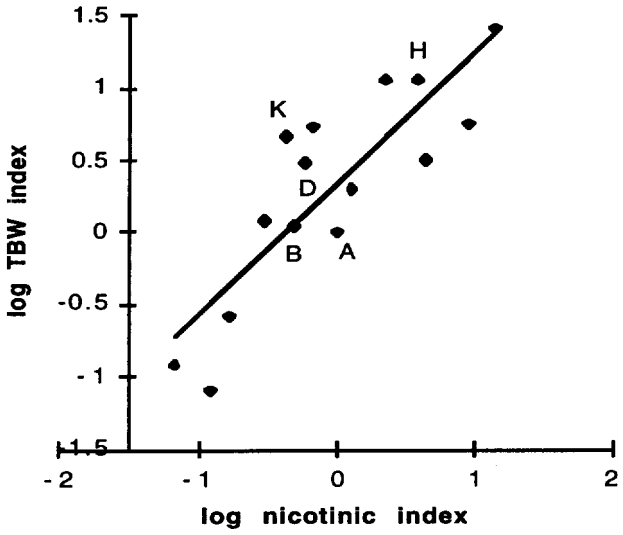


Figure 6. The correlation between an index of potency in activating nicotinic receptors in isolated cockroach neurons and an index of tobacco budworm activity, for a number of natural spinosyns (labeled points, structures in Sparks *et al.*, 1995, 1996) and synthetic spinosoids. Larger values of both indices denote lower potency.

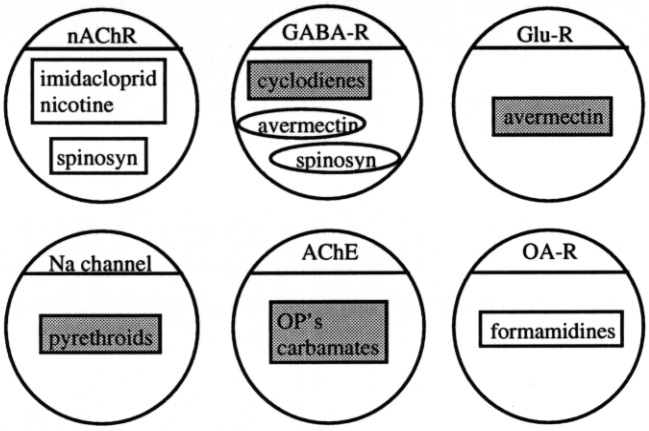


Figure 7. Major insect control target sites in the nervous system. The six proteins (circles) known to be targets for commercial insect control agents are the nAChR (nicotininic acetylcholine receptor), GABA-R, (γ-aminobutyric acid receptor), Glu-R (glutamate receptor), sodium (Na) channel, AChE (acetylcholinesterase) and OA-R (octopamine receptor). Putative target sites of well-established insecticidal importance are shown as boxes, while sites of questionable significance are shown as ovals. Sites where resistance occurs in at least one species are shaded.