CHEMISTRY AND BIOLOGY OF THE SPINOSYNS: COMPONENTS OF SPINOSAD (TRACER®), THE FIRST ENTRY INTO DOWELANCO'S NATURALYTE CLASS OF INSECT CONTROL PRODUCTS

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Abstract

The spinosyns are a new genre of fermentation-derived molecules that contain a 12-membered macrocyclic lactone in a unique tetracyclic ring. More than 20 spinosyns (A-Y) have been identified and result from variations in substitution patterns on the two sugars (forosamine and 2',3',4'-tri-O-methylrhamnose) and the tetracyclic ring system. Changes in the substitution pattern on the forosamine nitrogen have little effect on the activity of the spinosyns to neonate tobacco budworm larvae, while loss of a methyl group from the 2', 3' or 4' positions of the 2',3',4'-tri-O-methylrhamnose results in at least a 10-fold reduction in biological activity. Loss of a methyl group at C16 or C21 also reduces activity to neonate tobacco budworm larvae, while addition of a methyl group at C6 either has little effect or may slightly improve activity. The most active spinosyn identified to date to neonate tobacco budworm larvae is spinosyn A, which is also the principal component of spinosad (**Tracer**®), a naturally occurring mixture spinosyn A and spinosyn D.

Introduction

Insecticide resistance is a central theme in cotton insect control, especially for the tobacco budworm (Sparks 1980, Sparks et al. 1993). Managing insecticide resistance in the tobacco budworm, *Heliothis virescens*, and other species of *Heliothis / Helicoverpa*, has become a dominant focus in cotton in the United States and much of the world (Sparks et al. 1993, Leonard et al. 1995). Fundamental to managing resistance is the availability of safe and effective means to control target insects. In many parts of the U.S. and world, the options for controlling *Heliothis / Helicoverpa* are increasingly limited (Sparks et al. 1993, Martin et al. 1994, Leonard et al. 1995, Ahmad et al. 1995). Spinosad (**Tracer**®), the first entry into DowElanco's Naturalyte class of insect control products, presents a potential new option for the control of these insect pests. Spinosad represents a whole new genre of naturally derived insect control agents that possess pyrethroid levels of activity against a variety of lepidopterous cotton insect pests, including the tobacco budworm (Sparks et al. 1995, Thompson et al. 1995a,b). In addition, spinosad also exhibits exceptionally favorable environmental and mammalian toxicity profiles (Sparks et al. 1995, Thompson et al. 1995a,b), as well as no apparent cross-resistance to currently available insect control agents (Sparks et al. 1995). Spinosad is a naturally occurring mixture comprised of two active components, spinosyn A (about 85%) and spinosyn D (about 15%) (Fig. 1).

In the early 1980s a directed fermentation screening program undertaken by Lilly Research Laboratories led to the discovery of *Saccharopolyspora spinosa*, a new species of *Actinomyces* (Mertz and Yao 1990), extracts of which caused mortality to mosquito larvae (Kirst et al. 1992) and southern armyworm larvae (Sparks et al. 1995). The insect control activity was due to a family of new, unique macrocyclic lactones (Kirst et al. 1992) called the spinosyns (Sparks et al. 1995). A variety of techniques were used to establish the structure and stereochemistry of spinosyn A including mass spectrometry, extensive NMR spectroscopy, x-ray crystallographic analysis and hydrolysis of the forosamine sugar to establish absolute configuration (Kirst et al. 1992). As shown for spinosyn A, the spinosyns are a family of fermentation-derived natural products that are characterized by the presence of 1. amino sugar - forosamine, 2. neutral sugar - 2',3',4'--tri-O-methyl rhamnose and 3. a unique tetracyclic ring system containing a 12-membered macrocyclic lactone (Fig. 2).

The original parent (wild type: WT) strain of *S. spinosa* produced a number of spinosyns (A - J), but only in very minute quantities. Consequently, Lilly Research Laboratories began a program of strain improvement to increase the yield. One outgrowth of the program was the identification of several mutant strains, possessing a non-functional 2'- or 3'- or 4'-*O*-methyltransferase. These mutant strains produced a variety of spinosyns, some identical to those from the WT strain (i.e. spinosyns J and H), but most of the others were new. The spinosyns from a mutant strain possessing a non-functional 2'-*O*-methyltransferase (spinosyn H mutant) included spinosyns H, Q, R, S & T, while those from a mutant strain possessing a non-functional 3'-*O*-methyltransferase (spinosyn J mutant) included spinosyns J, L, M & N (Table 1). The third mutant strain, possessing a non-functional 4'-*O*-methyltransferase (spinosyn K mutant) produced spinosyns K , O & Y. Sinefungin, a methyltransferase inhibitor (Chen et al. 1989) was found to specifically block the 4'-*O*-methyltransferase during the fermentation process of the WT strain, and was used as an alternative method to generate spinosyns K & O, as well as several other new

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spinosyns, P, U, V & W, when used in conjunction with the spinosyn H (non-functional 2'-*O*-methyl-transferase) and spinosyn J (non-functional 3'-*O*-methyltransferase) mutant strains (Table 1).

The variations observed in the spinosyns center around;

1. methylation of the forosamine nitrogen
2. Presence or absence of O-methyl groups at the 2', 3' or 4' positions of the rhamnose sugar
3. Presence or absence a methyl group in the C6 and C16 positions, and presence of a methyl or ethyl group at the C21 position of the tetracyclic ring system. To date more than 20 spinosyns have been identified.

Materials And Methods

Compounds: Technical samples (95-99%) of the spinosyns and spinosad were provided by DowElanco and LRL. Analytical grade (99%) samples of cypermethrin were purchased from Chem Service.

Insects: The DowElanco laboratory colony of susceptible tobacco budworm larvae was started from individuals provided by the USDA laboratory in Stoneville, MS. All insects were reared on an artificial diet at 27°C ± 2°C, 50% rh., with a 14L:10D photoperiod.

Bioassay: A drench bioassay was used to evaluate biological activity (Sparks et al. 1995). Briefly, newly hatched (<6 hrs.) tobacco budworm larvae were placed on a filter paper. One ml of test solution was pipetted directly onto the larvae and the filter paper. Immediately after treatment the treated larvae and filter paper were placed in a plastic petri dish, and after 1 hr. a small untreated square of artificial diet was placed in the dish as a food source. Controls were treated with the solvent only. Mortality was determined at 24 hr. after treatment. The dose-response data (minimum of four (usually five) doses) with 20-40 larvae per dose were analyzed by probit analysis after Finney (1971). Topical bioassays were performed on third instar (20-30 mg) *H. virescens* larvae as described previously (Leonard et al. 1988).

Results and Discussion

Very simple changes in structure can profoundly influence the activity of the spinosyns towards neonate tobacco budworm larvae. Among the spinosyns from the WT strain, spinosyns H & J are both missing *O*-methyl groups in the rhamnose ring which reduces (10-fold and >200-fold, respectively) activity compared to spinosyn A (Table 1). In contrast, the presence or absence of *N*-methyl groups on the forosamine nitrogen (spinosyns B & C) or methyl at C6 (spinosyn D) do little to alter the biological activity, while loss of a methyl group at C16 or C21 (spinosyns E & F) reduces activity (Table 1). Available information suggests that spinosyns D, E & F all arise from the appropriate interchanges of acetate with propionate at suitable points during the biosynthesis of the spinosyns.

The spinosyn H mutant (non-functional 2'-*O*-methyltransferase) produced spinosyn H, its C6-methyl analog Q, and spinosyns R, S & T (Table 1). With the exception of spinosyn Q (LC_{50} = 0.5 ppm) which exhibited activity only slightly less than that of spinosyn A (LC_{50} = 0.31 ppm), all of the spinosyns from this mutant strain were much less active than spinosyn A. The spinosyn J mutant strain (non-functional 3'-*O*-methyltransferase) produced spinosyn J, its C6-methyl analog, spinosyn L, and spinosyns M & N, all of which were much less active than spinosyn A (Table 1). The spinosyn K mutant (non-functional 4'-*O*-methyltransferase) produced spinosyn K, its C6-methyl analog, spinosyn O, and spinosyn Y (Table 1). Spinosyns K & O were moderately active (LC_{50} = 1.4-3.5 ppm) while spinosyn Y was much less active (LC_{50} = 22 ppm) (Table 1). The new spinosyns resulting from the fermentation of the H and J mutants with sinefungin were no more active than the respective H and J analogs (Table 1). Thus, all of the new spinosyns are less active towards larvae of the tobacco budworm compared to spinosyn A, the principle component of spinosad.

Bioassays of spinosyn A using a standard topical bioassay produce a 72hr. LD_{50} of 1.3- 2.4 µg/g in our colony of tobacco budworms, a range that is quite similar to that observed for other tobacco budworm colonies (Leonard et al. 1996). This level of activity is comparable to a variety of pyrethroids (data averaged from the published reports of Dowd and Sparks 1988, Graves et al. 1964, 1967, Leonard et al. 1988, Nosky et al. 1980, Polazzo 1978, Rose and Sparks 1984, Sparks et al. 1982, Whitten and Bull 1974 and our own bioassays), and is certainly more effective than a wide range of organophosphorus, carbamate and other insect control agents (Fig. 2; see also Sparks et al. 1995). In a variety of bioassays, spinosyn A (the principle component of spinosad) is comparable to, and in some cases superior, to pyrethroids such as cypermethrin (Sparks et al. 1995). In general, some pyrethroids such as cypermethrin may possess somewhat better contact activity than spinosyn A, but spinosyn A is generally more active than these pyrethroids in assays that have an oral component (Sparks et al 1995).

Mode of Action: A number of modes of action for insect control agents are known. Extensive studies at DowElanco have shown that spinosyn A acts on the insect central nervous system to increase spontaneous activity via action on certain neurotransmitter receptors. To date, information is consistent with spinosyn A acting at a novel site(s) on these neurotransmitter receptors. Thus, spinosyn A appears to have a mode of action that is unique among insect control agents.

Summary

Although more than 20 spinosyns have been identified, none, to date, is more effective against tobacco budworm larvae than spinosyn A, the principal component of spinosad (Tracer®). Small changes in the structure of the spinosyns can result in large changes in biological activity, especially modifications to the tetracyclic ring and the rhamnose sugar. These novel compounds represent a new genre of unique, naturally derived insect control agents that possess pyrethroid - levels of activity, an excellent toxicological and environmental profile, and do not appear to be cross-resistant to any of the currently available insect control agents (Sparks et al. 1995).

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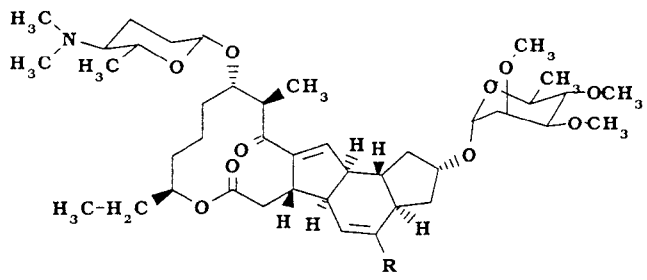
Table 1 - Structures, Sources and Neonate TBW Toxicity of the Spinosyns Compared to Spinosyn A

R1[a]	R2	R21	R16	R6	R2'	R3'	R4'	Sinfng[b]	LC_{50}[c]
Spinosyns from wild type									
A	**Me**	**Me**	**Et**	**Me**	**H**	**OMe**	**OMe**	**OMe**	o	**0.3**
B	***H***	-[d]	-	-	-	-	-	-	o	0.4
C	***H***	***H***	-	-	-	-	-	-	o	0.8
D	-	-	-	-	***Me***	-	-	-	o	0.8
E	-	-	***Me***	-	-	-	-	-	o	4.6
F	-	-	-	***H***	-	-	-	-	o	4.5
G[e]	***Me***	***Me***	-	-	-	-	-	-	o	7.1
H	-	-	-	-	-	***OH***	-	-	o	5.7
J	-	-	-	-	-	-	***OH***	-	o	>80
Spinosyns from H mutant: non-functional 2'-O-methyltransferase									
H	-	-	-	-	-	***OH***	-	-	o	5.7
Q	-	-	-	-	***Me***	***OH***	-	-	o	0.5
R	***H***	-	-	-	-	***OH***	-	-	o	14.5
S	-	-	***Me***	-	-	***OH***	-	-	o	53
T	-	-	-	-	-	***OH***	***OH***	-	o	>64
Spinosyns from J mutant: non-functional 3'-O-methyltransferase									
J	-	-	-	-	-	-	***OH***	-	o	>80
L	-	-	-	-	***Me***	-	***OH***	-	o	26
M	***H***	-	-	-	-	-	***OH***	-	o	22.6
N	***H***	-	-	-	***Me***	-	***OH***	-	o	40
Spinosyns from K mutant: non-functional 4'-O-methyltransferase									
K	-	-	-	-	-	-	-	***OH***	o	3.5
O	-	-	-	-	***Me***	-	-	***OH***	o	1.4
Y	-	-	***Me***	-	-	-	-	***OH***	o	20
Spinosyns from H and J mutants: non-functional 2' or 3'-O-methyltransferase - in combination with sinefungin									
U	-	-	-	-	-	***OH***	-	***OH***	+	22
V	-	-	-	-	***Me***	***OH***	-	***OH***	+	17
P	-	-	-	-	-	-	***OH***	***OH***	+	>64
W	-	-	-	-	***Me***	-	***OH***	***OH***	+	>64

a) See Fig. 2 for location of the R-groups on spinosyn structure.
b) Sinefungin (o = absent, + = present)
c) ppm
d) dash (-) indicates that the substitution is the same as for spinosyn A.
e) 4''-*N-epi*



spinosyn A R = H
spinosyn D R = Methyl

Figure 1. Structure of spinosad (Tracer®) = 85% spinosyn A, 15% spinosyn D