# Relative Effectiveness of Selected Stilbene Optical Brighteners as Enhancers of the Beet Armyworm (Lepidoptera: Noctuidae) Nuclear Polyhedrosis Virus

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**ABSTRACT** The addition of a stilbene optical brightener, Tinopal LPW, at 1% concentration (wt:wt) significantly reduced the $LC_{50}$ of the beet armyworm nuclear polyhedrosis virus (SeMNPV) from 2.9 PIB/mm$^2$ to 0.02 PIB/mm$^2$. Moreover, the $LT_{50}$ of SeMNPV was reduced by 34% by the addition of Tinopal LPW. Seven other structurally related stilbene brighteners were also tested as viral enhancers. Five of these brighteners (Tinopal LPW, Blankophor BBH, Blankophor HRS, Blankophor P167, and Blankophor RKH) reduced $LD_{50}$s, whereas three brighteners (Blankophor BSU, Blankophor DML, and Blankophor LPG) had little effect. Among the active brighteners, $LC_{50}$s were reduced by 10.5-fold (Blankophor P167), 52.4-fold (Blankophor RKH), 87.3-fold Tinopal LPW), 131-fold (Blankophor BBH), and >400-fold (Blankophor HRS). $LT_{50}$s were also decreased by the addition of Blankophor BBH, Blankophor P167, and Blankophor RKH, but were increased by the addition of Blankophor BSU, Blankophor DMLO, and Blankophor LPG to SeMNPV suspensions.

**KEY WORDS** *Spodoptera exigua,* optical brighteners, virus enhancement

THE BEET ARMYWORM, *Spodoptera exigua* (Hübner), is polyphagous and feeds on many economically important crops such as cabbage, celery, lettuce, cotton, and chrysanthemum (Smith 1989, Kolodny-Hirsch et al. 1993, Diawara et al. 1994, East et al. 1994). In both the United States and the Netherlands, *S. exigua* is a serious pest of greenhouse-grown crops, and a nuclear polyhedrosis virus (SeMNPV) has been used to control beet armyworm populations (Vlak et al. 1982, Smits et al. 1987). SeMNPV has also been an effective microbial control agent against *S. exigua* on lettuce (Gelernter et al. 1986), tomatoes, peppers, and garbanzo beans (Kolodny–Hirsch et al. 1993). Subsequent research by Kolodny–Hirsch and colleagues led to the registration of the virus (SPOD-X, Thermo Trilogy, Columbia, MD).

During the past several years we have been conducting research on optical brighteners as activity enhancers of insect viruses. Initial research involved the gypsy moth, *Lymantria dispar* (L.), nuclear polyhedrosis virus (LdMNPV) (Shapiro and Robertson 1992, Shapiro et al. 1992), but was subsequently expanded to include NPVs of the corn earworm, *Helicoverpa zea* (Boddie) (Shapiro and Vaughn 1995), and the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Hamm and Shapiro 1992, Shapiro and Hamm 1999) and their insect hosts. Since the initial discovery that stilbene optical brighteners could enhance the activity of LdMNPV, other researchers have subsequently demonstrated that selected stilbene brighteners could enhance the biological activities of NPVs against such economically important lepidopteran pests as the tobacco budworm, *Heliothis virescens* (F.) (Zou and Young 1994, Vail et al. 1996), soybean looper, *Pseudoplusia includens* (Walker) (Zou and Young 1996), cabbage looper, *Trichoplusia ni* (Hübner) (Washburn et al. 1998), and the western spruce budworm, *Choristoneura occidentalis* Freeman (Li and Otvos 1999a, 1999b). The purpose of our study was to determine whether brighteners that act as activity enhancers for NPVs against the corn earworm, fall armyworm, and gypsy moth could also act as enhancers against the beet armyworm and to determine whether function (=activity enhancement) could be related to chemical structure among structurally similar brighteners.

## Materials and Methods

**Insects and Viral Inoculum.** The colonized strain of *S. exigua,* established and maintained at USDA-ARS, Tifton, GA, was used for this study. Several egg masses were placed in 180-ml containers (Sweetheart Cup, Chicago, IL) and hatching larvae fed on a diet designed for the gypsy moth, *Lymantria dispar* (L.) (Bell et al. 1981). Larvae were maintained in a growth chamber at 29°C, 50% RH, and a photoperiod of 12:12 (L:D) h until use. The beet armyworm nuclear polyhedrosis virus (SeMNPV) was obtained from the Insect Biocontrol Laboratory (USDA-ARS, Beltsville, MD) virus collection as an aqueous suspension. For production of fresh NPV, SeMNPV was diluted to a concentration of $10^6$ polyhedral inclusion bodies (PIB) and 100 larvae (6 d old) were infected (10 larvae per 180-ml con-

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**Table 1. Effect of Tinopal LPW (1%) on the activity of *S. exigua* NPV (SeMNPV)**

| Treatment[a] | Slope (±SEM) | LC Levels (95% CL)[b] | | | | |
|---|---|---|---|---|---|---|
| | | 10 | 30 | 50 | 70 | 90 |
| SeMNPV only | 1.84 ± 0.15 | 0.55 (0.35–1.26) | 1.43 (0.75–3.35) | 2.87 (1.09–7.25) | 5.93 (2.79–15.30) | 17.48 (7.02–45.07) |
| SeMNPV/Tinopal LPW | 1.85 ± 0.06 | 0.007 (0.003–0.01) | 0.01 (0.007–0.02) | 0.02 (0.01–0.03) | 0.03 (0.01–0.04) | 0.04 (0.03–0.09) |
| Activity ratio[c] | | 79 | 143 | 143 | 198 | 437 |

[a] Number of PIBs/mm$^2$ of diet surface per cup required to produce 10, 30, 50, 70, and 90% virus-caused mortality among beet armyworm larvae.

[b] Six replicates; 10 larvae per NPV concentration per treatment per replicate; 10 untreated larvae per replicate; 10 Tinopal LPW only-treated larvae per replicate.

[c] Activity ratio, LC level for SeMNPV only divided by LC level for SeMNPV/Tinopal LPW.

tainer; 1 ml of virus suspension per container). Larvae were reared at 29°C, 50% RH, and a photoperiod of 12:12 (L:D) h for 10 d. All virus-killed larvae were collected and frozen (−20°C until usage).

**Bioassays.** The beet armyworm nuclear polyhedrosis virus (SeMNPV) was extracted from virus-killed larvae using standardized methodology (Shapiro et al. 1981). The insects were homogenized (e.g., each gram of insect tissue was blended in 9 gm distilled water) and filtered through coarse cheesecloth, and the filtrate was collected (=stock virus suspension). One milliliter of the stock suspension was diluted in 9 ml distilled water. A sample was removed by Pasteur pipette and the concentration of this suspension (1:10) was determined using a double-line hemacytometer with improved Neubauer ruling and phase microscopy (430× magnification). Dilutions were made from the stock suspension either in distilled water (standard treatment) or in brightener (test treatment) to produce concentrations ranging from $10^1$ to $10^6$ PIB/ml and 1.0 ml of a virus suspension was pipetted onto the diet surface (180-ml container; 4,770 mm$^2$ = surface area). In addition, both untreated controls and test controls (1.0% brightener only) were used to determine effects on beet armyworm larvae.

Ten second instars (4 d old) were placed in each container and were reared for 14 d at 29°C, 50% RH, and a photoperiod of 12:12 (L:D) h. Tests were repeated six times with 10 larvae per virus dilution per treatment per replicate and 10 untreated larvae and 10 brightener-treated larvae per treatment. Mortality was assessed initially at day 3 after treatment, then daily through day 7, and every 2–3 d thereafter until day 14. In the initial test, Tinopal LPW (1% wt:wt) was tested as an enhancer to SeMNPV. Thereafter, seven structurally stilbene brighteners were also tested and compared with Tinopal LPW(1% wt:wt).

**Optical Brighteners.** Eight stilbene optical brighteners were obtained as technical powders. All materials were diluted in distilled water and were tested at a 1% concentration (wt:wt). The seven Blankophor brighteners obtained from Bayer, Pittsburgh, PA) were as follows: Blankophor BBH (CAS # 4404-43-7), Blankophor BSU (CAS # 68971-49-3), Blankophor DML (CAS # 16090-02-1), Blankophor HRS (CAS # 61951-69-7), Blankophor LPG (CAS # 133-66–4), Plankophor P167 (CAS # 1670-24-9). Tinopal LPW (=Calcofluor M2R) (CAS # 4404-43-7) was obtained from Sigma, St. Louis, MO. These brighteners were selected for comparison, because they are all bistriazinyl derivatives of 4,4′-diamino-2,2-disulfonic acid (Argauer and Shapiro 1997). From the information supplied by the manufacturer, Blankophors BBH, DML, LPG, and RKH also contained formaldehyde polymers with sulfonated 1,1′-oxybis(methylbenzene) sodium salts.

**Statistical Methods.** Concentration-mortality and time-mortality regressions were estimated by probit analysis (LeOra Software 1987) to monitor the biological activities of SeMNPV with a without brightener. Failure of 95% CL to overlap was used as the criterion for significant differences at $LC_{50}$.

## Results

The addition of Tinopal LPW (1%) significantly reduced the $LC_{50}$ of SeMNPV ≈144-fold to 0.02 PIB/mm$^2$ (Table 1) and the $LC_{90}$ ≈444-fold to 0.04 PIB/mm$^2$. In the SeMNPV-only treatment, ≈6.1 times more virus was required to increase virus-caused mortality from 50 to 90%, whereas only two times more virus was required to increase mortality from 50 to 90% in the SeMNPV/LPW treatment (Table 1). Fifty percent of the test larvae died within 4.4 d when treated with SeMNPV alone, whereas the same percentage of SeMNPV/LPW-treated larvae died within 3.0 d. Thus, the addition of Tinopal LPW resulted in a ≈30% reduction in the rate of kill (Table 3).

Five of the eight stilbene optical brighteners acted as activity enhancers for SeMNPV. $LC_{50}$s were reduced ≈10.5-fold by Blankophor P167, ≈50-fold by Blankophor RKH, ≈90-fold by Tinopal LPW, ≈140-fold by Blankophor BBH, and >400-fold by Blankophor HRS (Table 2). When any of these brighteners were used in combination with SeMNPV, only two to three times more virus was required to increase virus-caused larval kill from 50 to 90%. For these brighteners, $LC_{90}$s ranged from 0.01 (Blankophor HRS) to 0.71 PIB/mm$^2$ (Blankophor P167). In general, as the efficiency of the brightener increased as an activity enhancer, the amount of PIBs required to increase mortality from 50 to 90% decreased (Table 2). The $LT_{50}$s were reduced by ≈20% by the addition or Tinopal LPW or Blankophor HRS and by ≈30% by the addition of Blankophor BBH or Blankophor RKH but were unaffected by the addition of Blankophor P167.

Three of the eight brighteners, however, did not act as enhancers for SeMNPV (e.g., Blankophor BSU,

**Table 2. Effects of stilbene optical brighteners on the activity of SeMNPV:$LC_{50}$, $LC_{90}$**

| Treatment[a] | Slope ± SEM | $LC_{50}$[b] (95% CL) | LC 90[b] (95% CL) | Activity ratio[c] |
|---|---|---|---|---|
| SeMNPV | 1.83 ± 0.02 | 2.62 (0.99–7.09) | 16.00 (6.02–43.23) | 1.00 |
| SeMNPV/LPW | 2.01 ± 0.05 | 0.03 (0.01–0.06) | 0.06 (0.04–0.13) | 87.33 |
| SeMNPV/BBH | 1.74 ± 0.03 | 0.02 (0.01–0.05) | 0.05 (0.03–0.13) | 131.00 |
| SeMNPV/P167 | 1.41 ± 0.02 | 0.25 (0.09–0.69) | 0.71 (0.19–1.51) | 10.48 |
| SeMNPV/HRS | 1.84 ± 0.04 | 0.007 (0.004–0.01) | 0.01 (0.007–0.03) | 374.29 |
| SeMNPV/RKH | 1.71 ± 0.04 | 0.05 (0.02–0.08) | 0.14 (0.05–0.22) | 52.40 |
| SeMNPV/LPG | 1.45 ± 0.02 | 3.56 (1.48–8.95) | 30.61 (12.83–77.15) | 0.74 |
| SeMNPV/DML | 1.38 ± 0.02 | 3.04 (1.17–8.76) | 24.82 (9.60–71.59) | 0.86 |
| SeMNPV/BSU | 1.45 ± 0.02 | 1.84 (0.65–5.32) | 15.37 (5.41–44.36) | 1.42 |

[a] SeMNPV was diluted in distilled water (standard) or in 1% optical brightener. SeMNPV was used at final concentrations of 10 to 1 million PIBs per cup.

[b] $LC_{50}$s and $LC_{90}$s are expressed as PIB/ml per cup; 10 larvae per virus concentration per treatment per replicate; 10 untreated larvae and 10 larvae exposed to each brightener only were used in each replicate. No mortality was observed in any control or brightener-only treatment. To obtain $LC_{50}$s or $LC_{90}$s in terms of PIB per $mm^2$, divide $LC_{50}$s or $LC_{90}$s by 4,770.

[c] Activity ratio, LC level for SeMNPV only divided by LC level for SeMNPV/Tinopal LPW.

Blankophor DML, Blankophor LPG) (Table 2). Eight- to ninefold more virus was required to increase the virus kill from 50 to 90% when these brighteners were used with SeMNPV, as compared with a sixfold increase when SeMNPV was used alone (Table 2). Moreover, the time required for 50% of the test larvae to die from virosis ($LT_{50}$) was increased by ≈20% by Blankophors BSU, DML, and LPG (Table 2).

## Discussion

During the past several years, we have been determining the enhancement capabilities of stilbene optical brighteners in different host-virus systems (Hamm and Shapiro 1992, Shapiro and Robertson 1992, Shapiro and Vaughn 1995, Hamm and Chandler 1996). For many of these studies, Tinopal LPW (=Calcofluor M2R; =Fluorescent Brightener 28) has been used as the standard. In the current study, the addition of Tinopal LPW significantly enhanced the activity of SeMNPV in terms of both virus concentration (Hamm and Chandler 1996) (Tables 1 and 2) and in time of kill (Table 3). Whereas, ≈55 PIB/$mm^2$ was required to cause a lethal infection among larvae exposed to SeMNPV only (=$LC_{10}$), only 0.007 PIB/$mm^2$ was needed in the SeMNPV/Tinopal LPW treatment to produce the same level of mortality. To cause 90% mortality among larvae exposed to SeMNV, only >17 PIB/$mm^2$ was required. Among larvae exposed to the SeMNPV/Tinopal LPW combination, only ≈.04 PIB/$mm^2$ was needed to produce 90% virus-caused mortality (Table 1). Thus, Tinopal LPW not only decreased the amounts of virus required to produce lethal infections but also lessened the amount of PIBs needed to increase virus-caused from mortalities from 10 to 50% and from 50 to 90%. Moreover, the addition of Tinopal LPW also influenced the speed of kill, because beet armyworm larvae died sooner than insects exposed to SeMNPV alone.

**Table 3. Effect of stilbene optical brighteners on the speed of kill of SeMNPV**

| Treatment[a] | LT 50 (95% CL) in days[b] | Relative speed of kill (%)[c] |
|---|---|---|
| SeMNPV | 4.28 (3.60–5.10) | — |
| SeMNPV/LPW | 3.37 (2.91–3.92) | −21.3 |
| SeMNPV/BBH | 3.03 (2.56–3.63) | −29.2 |
| SeMNPV/P167 | 3.74 (3.17–4.42) | −12.6 |
| SeMNPV/HRS | 3.36 (2.76–4.29) | −21.5 |
| SeMNPV/RKH | 2.95 (2.47–3.52) | −31.1 |
| SeMNPV/LPG | 5.22 (4.27–6.40) | +22.0 |
| SeMNPV/DML | 5.28 (4.32–6.50) | +23.4 |
| SeMNPV/BSU | 5.01 (4.18–6.00) | +17.1 |

[a] Optical brighteners were used at 1% (wt:wt) concentration.

[b] $LT_{50}$s were taken for a virus concentration of $10^6$ million PIBs per cup and were averaged (six replicates) for each treatment.

[c] The $LT_{50}$ for SeMNPV was used as the standard and all other $LT_{50}$s were compared to the standard. Negative values indicate that the test treatment resulted in faster kill than produced by the standard treatment (SeMNPV alone). Positive values indicate that the test treatment resulted in slower kill than the standard treatment (SeMNPV alone).

The Blankophor optical brighteners were selected for testing, because they are structurally similar to our standard brightener Tinopal LPW. All brighteners are bistriazinyl derivatives of 4,4′ diaminostilbene 2,2- disulfonic acid but have different chemical groups attached to the stilbene backbone (Argauer and Shapiro 1997). Both Tinopal LPW and Blankophor BBH possess the same CAS number (CAS #4404-43-7) and each can be converted into the other. Tinopal LPW (pH 9.5, 1%) can be converted to Blankophor LPW (pH 6.9, 1%) by acid treatment and Blankophor BBH can be converted into Tinopal LPW with alkali treatment with no loss in enhancement activity. Despite similarities in chemical structure, differences in the ability of enhancement activity were found among the eight brighteners (Tables 2 and 3). For SeMNPV, Tinopal LPW, Blankophor BBH, Blankophor HRS, Blankophor P167, and Blankophor RKH acted as enhancers, whereas Blankophor BSU, Blankophor DML, and Blankophor LPG did not enhance the activity of SeMNPV.

The same stilbene brighteners have been tested as enhancers for HzSNPV, LdMNPV, and SfMNPV in their homologous hosts. These studies were initiated to determine whether a brightener that acted as an enhancer for a given host-NPV system would also act as an enhancer for other host-NPV systems or whether activity varied from host to host. In general, similar

results were obtained in all four host-virus systems and indicated that the brighteners that were active in one system (Tinopal LPW, Blankophor BBH, Blankophor HRS, Blankophor P167, Blankophor RKH) were active in all systems tested. Differences in the relative rankings of active brighteners might, however vary from one host-virus system to another. For example Blankophor HRS was most active in the beet armyworm-SeMNPV system but was not as active as Tinopal LPW, Blankophor BBH, Blankophor P167, and Blankophor RKH in the gypsy moth-LdMNPV system. Conversely, those brighteners that did not enhance activity in one system (Blankophor BSU, Blankophor DML, Blankophor LPG) tended not to be active in other systems. Thus, it would appear that a given brightener might be useful in different virus-host systems.

Currently, differences in enhancement activity among the structurally similar stilbene brighteners cannot be attributed to apparent differences in structure. In general, the greater the number of sulfonic acid groups the greater is the water solubility. Is this related to activity? In the beet armyworm-SeMNPV system, the most active brightener (Blankophor HRS) contains two sulfonic acid groups but a nonactive brightener (Blankophor BSU) contains six sulfonic acid groups. In the gypsy moth-LdMNPV system, the most active brighteners contain either two (Blankophor BBH, Blankophor RKH, Tinopal LPW) or four (Blankophor P167) sulfonic acid groups.

All brighteners contain R groups such as anilino, sulfanilic acid, or anilin-2,5-disulfonic acid. Some differences occur among the R' groups. For example, enhancers contain methylamino (Blankophor HRS, Blankophor RKH), or bis (hydroxyethyl) amino (Blankophor BBH, Blankophor P167) groups. Nonenhancers contain anilino (Blankophor LPG), morpholino (Blankophor DML), or bid (hydroxyethyl) amino (Blankophor BSU) groups. The pHs of the eight brighteners ranged from 6.9 to 9.5 and no apparent relationship could be found between pH and viral enhancement.

Research in this area has been very fruitful and has demonstrated the activity of these chemicals as both effective sunlight protectants (Shapiro 1992, Dougherty et al. 1996) and activity enhancers (Shapiro et al. 1992; Zou and Young 1994, 1996) in different host-virus systems. Although these studies served to highlight the potential usefulness of stilbene brighteners with insect pathogenic viruses to control insect pest populations, studies on the mode of action of these chemicals (Adams et al. 1994, Sheppard and Shapiro 1994, Dougherty et al. 1995, Kirkpatrick et al. 1998, Washburn et al. 1998, Wang and Granados 1997, 2000) are of great importance in determining the factors responsible for virus specificity, virus activity, as well as host response (susceptible versus nonsusceptible).

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