raphy on silica gel and the sterol mixture (285 mg) was obtained from the eluate with benzene-ethyl acetate (9:1). This was analyzed as trimethylsilyl (TMS) ethers by gas chromatography with an open-tubular glass capillary column coated with OV-17 (40 m) at 270 °C. As indicated in the figure, fucosterol (1.6%) and isofucosterol (0.8%) were detected, together with cholesterol (41.5%), campesterol (3.3%), stigmasterol (1.2%) and sitosterol (51.6%). To confirm this, the sterol mixture was treated with pyridine-acetic anhydride and the resulting acetate was subjected to TLC on a silver nitrate impregnated silica gel G plate. Development with hexane-benzene (4:1) 5 times separated fucosterol acetate ($R_f = 0.35$) from isofucosterol acetate ($R_f = 0.30$). Each fraction was saponified, derivatized to the TMS ethers and then analyzed with a gas chromatograph-mass spectrometer using 3% OV-17 on Chromosorb W HP (1.5 m) at 248 °C. Selected ion monitoring on m/z 484 (M^+), 469 ($M-CH_3$) and 386 (M-McLafferty fragment ion) and 129 gave prominent peaks at the retention time of 12.2 min (fucosterol TMS ether) or 12.9 min (isofucosterol TMS ether). These results clearly showed the presence of both fucosterol and isofucosterol in *Bombyx mori*. Separate experiments indicated that mulberry leaves, on which the insects were reared, contain no appreciable amount of these sterols.

We have previously identified both (24R, 28R)- and (24S, 28S)-fucosterol epoxide (V) and (VI) in *B. mori* as intermediates of fucosterol dealkylation[7,8]. These results, together with the findings that sitosterol (I) and clionasterol (VII) indifferently satisfied the sterol requirement of *B. mori*[7], appear to suggest a rather loose stereospecificity in sitosterol dealkylation. However, species differences in the stereochemical course seem also to be evident from the recent observations that isofucosterol but no fucosterol was identified in the honey bee, *Apis mellifera* fed synthetic diets[9], and *Tenebrio molitor* larvae converted (24R, 28S)-isofucosterol epoxide, but not its (24S, 28R)-diastereoisomer, into cholesterol[10].

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Chitin synthesis inhibiting insect growth regulators do not inhibit chitin synthase

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Summary. Tunicamycin, an antibiotic, and 5 insect growth regulators (diflubenzuron, Bay Sir 6874, Bay Sir 8514, CGA-19255 and Lilly 494) do not inhibit chitin synthase obtained from 4-day-old *Stomoxys calcitrans* pupae.

Since the introduction of insect growth regulators that putatively inhibit chitin production a number of articles have appeared that have dealt with their mode of action. Because diflubenzuron[2] (Dimilin, TH-6040; N-[[(4-chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide) was one of the first chitin synthesis inhibitors (CSI) reported[3-5] it has received the most attention. Although there is agreement that diflubenzuron disrupts the formation of insect cuticle there is disagreement as to the molecular basis for this.

Ishaaya and Casida[6] observed an increase in chitinase activity in tissues obtained from house fly larvae reared on diets containing diflubenzuron. This observation led them to suggest that the reduced amounts of chitin in diflubenzuron-treated insects was the result of enhanced chitinase activity. However Deul et al.[5] repeated the experiments using *Pieris brassicae* (L.) larvae and found no effect on chitinase activity, but chitin deposition was affected.

Other investigators have suggested that diflubenzuron acts directly on the chitin synthase (EC 2.4.1.16; UDP-2-acetyl-amino-2-deoxy-D-glucose chitin 4-β-acetamidodeoxyglucosyltransferase)[5,7-9] which is the terminal enzyme in the synthesis of chitin. These suggestions are based on in vivo and in vitro organ culture work that showed a buildup of UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) in tissues treated with certain benzoylphenylurea CSI. Because UDP-GlcNAc is the obligatory substrate for chitin synthase it was logical to assume that chitin synthase was inhibited by diflubenzuron and UDP-GlcNAc accumulated as a result. Although this is a logical explantation for the mode of action of diflubenzuron and other CSI, Van Eck[9] and Mayer et al.[10] suggested that caution should be exercised in interpreting results from in vivo and in vitro organ culture experiments. Such experiments, at best, can only give indirect evidence for a mode of action.

Meola and Mayer[11] showed that in diflubenzuron treated stable fly (*Stomoxys calcitrans*, L.) pupae chitin synthesis did not occur because adult epidermis was not produced; diflubenzuron appeared to inhibit the proliferation of imaginal epidermal cells. This observation indicated that a different mode of action was operating, but it did not eliminate the possibility that there could be a direct inhibitory effect of diflubenzuron on chitin synthase.

Recent work performed by Cohen and Casida[12] and Mayer et al.[13] provided evidence that diflubenzuron did not inhibit chitin synthase. Both groups were able to isolate the chitin synthase in cell-free preparations and demonstrate that diflubenzuron had no effect on the enzyme.

The purpose of the present communication is to extend the work of Mayer et al.[13] by testing other CSI.

Materials and methods. Chemicals[2]. Radiolabeled UDP-[glucosamine-6-^{3}H]GlcNAc (6.6 Ci/mmole) and Bray's scintillation cocktail were purchased from New England Nuclear, Boston, MA and UDP-GlcNAc purchased from Sigma Chemical Co., St. Louis, MO. Tunicamycin was

obtained from Dr J.D. Douros, Developmental Therapeutics Program, Chemotherapy, NCI, Bethesda, MD.

The CSI were gifts: Bay Sir 8514 (2-chloro-N-[[[4(trifluoromethoxy)-phenyl]amino]carbonyl]benzamide) and Bay Sir 6874 (2-chloro-N-[[[3,5-dichloro-4-(4-nitrophenoxy)-phenol]amino]carbonyl]benzamide), Mobay Chemical Corp., Kansas City, MO; EL-494 (N-[[[5-(4-bromophenyl)-6-methyl-2-pyrazinyl]amino]-carbonyl]-2,6-dichlorobenzamide), Lilly Research Labs, Indianapolis, IN; diflubenzuron (Dimilin®; TH-6040; N-[[(4-chlorophenyl)-amino]-carbonyl]-2,6-difluorobenzamide)], Thompson-Hayward Chemical Co., Kansas City, KS.

Enzyme preparation. Chitin synthase was obtained from stable fly pupae reared following the procedures of Mayer et al.[14]. When the pupae were 4 days old (i.e. 4 days from the white prepupal stage) they were homogenized in 4 volumes of 50 mM MOPS buffer (pH 7.0) containing 0.25 M sucrose, 2 mM dithioerythritol (DTE) and 1 mM EDTA. The homogenate was filtered through 2 layers of cheesecloth, then centrifuged at 600 × g for 10 min. The pellet was resuspended in 2 volumes of homogenization buffer and recentrifuged as before. The supernatants of the 1st and 2nd centrifugations were pooled and centrifuged at 10,000 × g for 10 min. The supernatant was discarded and the pellet washed once by resuspension in 2 volumes of buffer and recentrifugated at 10,000 × g for 10 min. Finally the supernatant was discarded and the pellet resuspended in 50 mM MOPS buffer (pH 7.0) containing 2.0 mM DTE (0.75 ml buffer per 2 g fresh tissue weight). Protein in this suspension was determined by the method of Bradford[15]. Preparative procedures were conducted at 5 °C.

Chitin synthase assays. The assay mixtures contained about 0.7 mg of resuspended 10,000 × g protein and enough 50 mM MOPS buffer (pH 7.0) with 2 mM DTE to bring the volume to 200 µl. The reactions were initiated with ^{3}H-UDP-GlcNAc (5 µM final concentration; about 300,000 cpm). With the exception of tunicamycin which was dissolved in 50 mM MOPS buffer (pH 7.0) all of the CSI were dissolved in dimethylsulfoxide (DMSO). Inhibitors (1 µl) were added 10 min prior to initiating the reaction. Controls consisted of solvent addition only. The reaction was conducted at 30 °C for 2 h in 12 × 75 mm culture tubes.

Reactions were terminated by the addition of 2 ml of 66% aqueous ethanol. The reaction mixture was then filtered, washed and the radioactivity measured as described in Mayer et al.[13]. There were 3-6 replicates for each experiment.

Effects of tunicamycin and various insect chitin synthesis inhibitors on *S. calcitrans* chitin synthase

Compound	Concentration (µM)	% Control activity
None	-	101
DMSO Control*	1 µl	100
Diflubenzuron	5	96
	20	104
Bay Sir 6874	5	98
	20	103
Bay Sir 8514	5	98
	20	107
CGA-19255	5	98
	20	109
Lily 494	5	89
	20	103
Tunicamycin	12	94
	120	94
O-time DMSO control		0

* The mean CPM were 26,165 ± 831 (SD).

Results and discussion. Although many investigators[4,7-9] have suggested that CSI interfere with cuticle production in insects by directly inhibiting chitin synthase we can find no evidence for this. The results in the table clearly show that all of the CSI tested have little or no effect on the ability of chitin synthase to form chitin. These data parallel the results of Cohen and Casida[12] who used a chitin synthase preparation from gut tissues of *Tribolium.* This chitin synthase is thought to be responsible for the synthesis of the peritrophic membrane. The findings presented here also reconfirm our previous work[13] and extends it to include several additional CSI. The chitin synthase preparation used in this work differs from that of Cohen and Casida[12] in that we believe the enzyme we isolated is responsible for the production of cuticle chitin in *S. calcitrans.* The results in the table show that tunicamycin has no effect on the chitin synthase. Even at concentrations in excess of 300 µM there was no effect (data not shown). Tunicamycin is known to inhibit N-acetylglucosamine transferases and/or glucosyltransferases in molds, bacteria and mammals by preventing the transfer of GlcNAc from UDP-GlcNAc to certain lipid acceptors[16,17]. *Neurospora crassa* chitin synthase was reported to be inhibited by tunicamycin[18], however, others have not seen this effect using chitin synthases from different sources[12,19].

Possibly CSI such as diflubenzuron prevent the formation of GlcNAc-pyrophosphoryl-dolichol as has been shown for tunicamycin[17]. Such a mechanism has been suggested previously by Marks and Sowa[20]. If such inhibition occurred it could explain the accumulation of UDP-GlcNAc found in insects treated with diflubenzuron[7-9] because this is an obligatory substrate for glucosyltransferase as well as chitin synthase. The possibility of inhibition of glucosyltransferases by CSI is currently under investigation in our laboratory.

At this time there is no conclusive evidence for any suggested mode of action for the CSI.

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2 This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the USDA, nor does it imply registration under FIFRA as amended.
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