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Chitin Synthetase of *Neurospora crassa*: Inhibition by Nikkomycin, Polyoxin B, and UDP

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Abstract. The inhibitory effects of nikkomycin, polyoxin B, and UDP were tested on particulate chitin synthetase activity (UDP-2-acetamido-2-deoxy-D-glucose: chitin-4-B-acetamidodeoxy-D-glucosyltransferase, E.C.2.4.1.16) from *Neurospora crassa*. Two approaches were used: (a) inhibitors were tested for their individual effects on chitin synthetase activity; (b) paired combinations of inhibitors were examined to establish whether the compounds affected inhibition by binding at the same enzyme site. The first method showed that the three compounds are linear competitive inhibitors, i.e., each competes directly with the substrate for binding. $K_{i\ app}$ values were: UDP, 0.8 mM; polyoxin B, 32 μM; and nikkomycin, 2 μM. The second method showed that the inhibitors compete with each other for binding; thus they bind at the same site. The pyrimidine nucleoside moiety of these inhibitors is an essential component for effective inhibition, but the potency of inhibition is critically dependent on the conformation of the side group attached to carbon 5 of the ribose sugar.

Nikkomycin and polyoxin are nucleoside-peptide antibiotics that have been shown to inhibit fungal growth [5, 7] and, specifically, to inhibit chitin synthetase (UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-B-acetamidodeoxy-D-glucosyltransferase, E.C.2.4.1.16). Chitin is a polysaccharide that is an essential component of the cell wall of most fungi, but does not occur in either humans or plants [20]; inhibition of the biosynthesis of chitin is, therefore, a target for antifungal antibiotics of both clinical and agricultural application. During the synthesis of chitin, the nucleotide moiety UDP is released from UDPGlcNAc. Some reports suggest that UDP affects the activity of chitin synthetase in vivo through competitive inhibition [14, 21]. Polyoxins and nikkomycins are structurally similar to UDPGlcNAc, the substrate for chitin synthetase, and act as competitive inhibitors [2, 5, 7, 12, 19].

Competitive inhibition can result from a number of possible enzyme–inhibitor interactions. The inhibitor may compete with a substrate for binding at the active site; alternatively, the inhibitor may affect enzyme activity by binding at one (or more) other locations and causing enzyme conformational changes that affect enzyme affinity for substrate. Since polyoxin, nikkomycin, and UDP are competitive inhibitors of chitin synthetase, the question arises as to how they affect their inhibition. More specifically, does each of the three interact with the enzyme at the same site? Yonetani and Theorell [27] have devised a method to establish the nature of enzyme–inhibitor interactions. According to this method, various concentrations of inhibitors are tested in pairwise combinations, then reciprocal velocities are plotted against the concentration of one of the inhibitors, producing a series of lines. If the two inhibitors being tested interact at the same site, then these lines will be parallel; if any other type of interaction occurs, the lines will cross at a point to the left of the ordinate. Information obtained from such experiments may then be related to inhibitor structure, and thus to active components of the catalytic site, and, eventually, to the details of enzyme catalysis. We have used this type of analysis to test the nature of the interactions occurring between UDP, polyoxin B, nikkomycin, and chitin synthetase.

Materials and Methods

Chemicals. Polyoxin B was purified from Polyoxin AL Wettable Powder (Kaken Chemical Co.) as described [24]. Nikkomycin Z was a generous gift of Bayer AG (Leverkusen, FRG). Radioactive UDP (1-^{14}C) GlcNAc was purchased from ICN. Biochemicals were purchased from Sigma Chemical Co. (St.

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Louis, MO); other chemicals were of reagent grade, and distilled water was used throughout.

Growth of *Neurospora crassa*. *Neurospora crassa* wild type (74-OR8-1a) was obtained from the Fungal Genetics Stock Center (Arcata, CA). Macroconidia (harvested from mycelial cultures grown on agar slants containing Vogel's minimal medium N [26] for 5–10 days at 25°C) were inoculated at 1×10^8 per flask into 250-ml Erlenmeyer flasks containing 50 ml of Vogel's minimal medium N and 1.5% (wt/vol) sucrose. Flasks were incubated at 25°C for 16 h with orbital shaking (140 rpm). Resulting hyphae were harvested by filtration onto Whatman no.1 filter paper and washed with fresh medium (2×15 ml). Each mycelial mat was weighed (one flask yielded 0.5–0.8 g wet weight) and frozen in solid CO_2.

Preparation of particulate cell-fraction. Frozen hyphae (stored at −70°C) were disrupted by grinding in a mortar and pestle containing glass beads (diameter 25 μm) and CO_2 powder. Ground hyphae (~ 1 g) were suspended in 5 ml of 50 m*M* HEPES, pH 7.5, containing 10 m*M* $MgCl_2$, and centrifuged briefly (30 *g*; 1 min) to remove glass beads and intact hyphae. Supernatants were centrifuged at 10,000 *g* (10 min; 2°C) and the resulting supernatants centrifuged at 100,000 *g* (ave) for 60 min at 2°C. Resulting pellets, containing particulate chitin synthetase activity, were resuspended in 500 μl 50 m*M* HEPES, pH 7.5, containing 10 m*M* $MgCl_2$ by brief sonication (~ 5s).

Chitin synthetase assay. Reaction mixtures (18 μl) contained 12.5 m*M* GlcNAc, 10 m*M* $MgCl_2$, 50 m*M* HEPES, pH 7.5, and various concentrations of substrate, UDP (1-^{14}C) GlcNAc. Mixtures were incubated at 30°C and reactions terminated by the addition of 5 μl glacial acetic acid. Chitin was separated from unreacted substrate by descending paper chromatography (Whatman no. 1) using 1 *M* ammonium acetate (pH 7.5)–ethanol (3:7) as solvent; insoluble radiolabeled chitin remained at the origins of chromatograms. The filter paper origins were excised and radioactivity determined by liquid scintillation counting. Enzyme kinetic data were processed using a nonlinear regression computer program (ROSFIT) described by Greco et al. [11] and an Apple II + microcomputer.

For mixed inhibitor experiments, the substrate concentration was constant and concentrations of polyoxin B, UDP, and nikkomycin were varied. Plots of reciprocal velocity against the concentration of one inhibitor resulted in a series of straight lines. Cleland [4] formulated the following equation to describe this situation:

$$1/v = 1/v_0 (1 + I/\mathrm{Ki} + J/\mathrm{Kj} + IJ/\alpha\ \mathrm{KiKj})$$

where v = inhibited rate, v_0 = uninhibited rate, I = concentration of the first inhibitor, J = the concentration of the second inhibitor, Ki and Kj = dissociation constants for *EI* and *EJ* (*E* = enzyme), and α is an interaction coefficient that indicates the extent to which the binding of *I* and *J* is mutually exclusive. If the lines converge, they will cross at the point $I = -\alpha\mathrm{Ki}$, $1/v = (1/v_0)(1 - \alpha)$. If $\alpha = 1$, there is no competition between the inhibitors for binding to the enzyme (i.e., they bind at different sites); if α is less than 1, the inhibitors interact synergistically; finally, an increasing value for α above 1 indicates increasing hindrance of the inhibitors owing to partial competition for the same site or sites on the enzyme.

Characterization of reaction product. The reaction product was tested for its sensitivity to chitinase digestion. Filter papers containing chromatographically immobile material (see previous section) were treated with chitinase (E.C.3.2.1.14; 200 μg/ml, purified as described in [18]) for 72 h at 25°C. Penicillin G (100 U/ml) and gentamycin sulfate (30 μg/ml) were included in the chitinase mixture in order to prevent bacterial growth. After incubation, the filter papers were removed, washed, and dried, and the amount of radioactivity solubilized by chitinase treatment was determined. Chitinase reaction mixtures were lyophilized, resuspended in water, and separated by paper chromatography (Whatman no. 1) using isopropanol–n-butanol–H_2O (14:2:4, vol/vol) as solvent. The rate of migration of the radioactive product of chitinase digestion was compared with that of diacetylchitobiose (diacetylchitobiose is the product of digestion by chitinase of authentic chitin [16]).

Results

Characterization of chitin synthetase activity. Young hyphae of *N. crassa* were harvested, disrupted, and fractionated by centrifugation; each fraction was assayed for chitin synthetase activity as described in *Materials and Methods*. The majority (> 60%) of the enzyme activity was recovered in 100,000 *g* pellets; this fraction was used for subsequent assays. Digestion of the ^{14}C-labeled reaction product with purified chitinase caused solubilization of more than 95% of the incorporated radioactivity; the chitinase digestion product comigrated with authentic diacetylchitobiose (data not shown), indicating that chitin was synthesized by the particulate enzyme preparation.

Chitin formation at 30°C was linear with respect to time (for at least 10 min) and with respect to protein concentration (up to 150 μg/assay). The optimum pH was pH 7.5 using HEPES buffer; HEPES was used instead of Tris HCl [1, 8, 23] because 40% more chitin synthetase activity was recovered using HEPES than using Tris (data not shown). Mg^{2+} (8–20 m*M*) caused a sixfold stimulation of activity (data not shown); 10 m*M* $MgCl_2$ was used routinely during fractionation and assay of enzyme activity. GlcNAc stimulated enzyme activity and caused a decrease in the apparent K_m for UDPGlcNAc (Fig. 1). This effect has been reported previously for *Neurospora* (23) and other fungi [9], but the reason for this result is not fully understood. The apparent K_m for UDPGlcNAc was ~ 3.9 m*M* (GlcNAc at 0 m*M*); addition of 3.1 m*M*, 6.25 m*M*, and 12.5 m*M* GlcNAc each resulted in $K_{m\ app}$ of ~

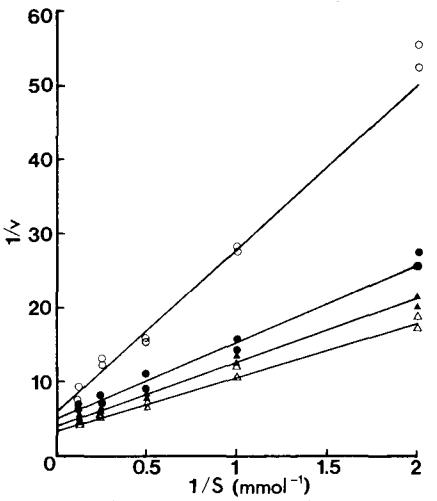

Fig. 1. Kinetic analysis of chitin synthetase activity. Resuspended high-speed particulate fractions were prepared as described, and samples containing 90 μg protein were added to reaction mixtures containing the indicated concentrations of UDPGlcNAc and the following GlcNAc concentrations: ○-○, 0 m*M*; ●-●, 3.1 m*M*; ▲-▲, 6.25 m*M*; △-△, 12.5 m*M*. The specific activity of UDP-^{14}C-GlcNAc was 1.8 × 10^8 dpm/mmol. Reaction mixtures were incubated for 0, 5, and 10 min at 30°C and the amount of chitin synthesized determined. Velocity (nmol product/min) was calculated and data plotted in the double reciprocal form using the ROSFIT program described in *Materials and Methods*. Each point represents an individual determination.

2.1 m*M*. The apparent activation constant, $K_{a\ app}$ GlcNAc, was found to be 3 m*M*.

Effects of inhibitors. UDP, polyoxin B, and nikkomycin inhibited chitin synthetase. Double reciprocal plots (Fig. 2a–c) indicated that each compound behaved as a competitive inhibitor (nomenclature of Cleland [3]) with respect to the substrate, UDPGlcNAc. Reciprocal slopes from Fig. 2a–c plotted against inhibitor concentration showed straight lines (data not shown), indicating that competitive inhibition was *linear* in each of the three cases. Estimates of $K_{i\ app}$, and standard deviations from one series of experiments are as follows: UDP, 0.8 ± 0.07 m*M*; polyoxin B, 32 ± 3 μ*M*; and nikkomycin, 2.0 ± 0.3 μ*M*. N-acetylglucosaminl-1-P had no effect on chitin synthetase activity at 2–20 m*M* in the presence of 12.5 m*M* GlcNAc (data not shown).

Effects of combinations of two inhibitors. The results from the last section revealed that polyoxin, UDP, and nikkomycin are linear competitive inhibitors with respect to UDPGlcNAc. However, to find out whether each of these compounds interacts with the enzyme at the same site, i.e., whether inhibitor binding is mutually exclusive or if the inhibitors interact in some way, pairwise combinations of inhibitors were added to reaction mixtures containing fixed substrate concentrations and enzyme activity measured. The results were plotted using a method described by Yonetani and Theorell [27]. The plots obtained for the three combinations UDP–polyoxin B (Fig. 3), UDP–nikkomycin (Fig. 4), and polyoxin B–nikkomycin (Fig. 5) each show a series of parallel lines. This indicates that polyoxin B and nikkomycin compete for the same site, as do polyoxin B and UDP, and nikkomycin and UDP.

Discussion

Chitin synthetase was first described in 1957 by Glaser and Brown [8], who found that the enzyme activity was localized primarily in high-speed pellets from homogenized hyphae of *N. crassa*. Since then, chitin synthetase has been the subject of intensive research, particularly in light of its importance in fungal cell-wall assembly (see Gooday and Trinci [10] for a review). Our results are in basic agreement with those of others: chitin synthetase activity is located mainly in high-speed particulate fractions of hyphal homogenates; enzyme activity is stimulated by GlcNAc and Mg^{2+}; and has a pH optimum of pH 7.5 and a K_m UDPGlcNAc of 2.1 m*M*. An interesting and noteworthy observation is that maximal enzyme activity was obtained using HEPES buffer (instead of the usual Tris buffer).

The main focus of this study was to determine the nature of the inhibition of chitin synthetase by UDP, nikkomycin, and polyoxin B. To do this, we adopted two lines of inquiry. The first is an extension of work by other authors [5, 7, 13–15] and concerns analysis of the type of competitive inhibition shown by these compounds. The second involves experiments using combinations of two inhibitors simultaneously, thus the effect of two inhibitors on enzyme inhibition may be observed. Such mixed-inhibitor experiments have been used to determine if inhibitors compete with each other for binding, i.e., whether or not they bind at the same location on the enzyme.

Using the first of the above approaches, we have shown that polyoxin B, nikkomycin, and UDP behave as *linear* competitive inhibitors of chitin synthetase activity (Fig. 2a–c), i.e., each competes directly with the substrate for binding to the enzyme. The $K_{i\ app}$ UDP (0.8 m*M*) is within the same range as that previously reported, e.g., 0.4 m*M* for

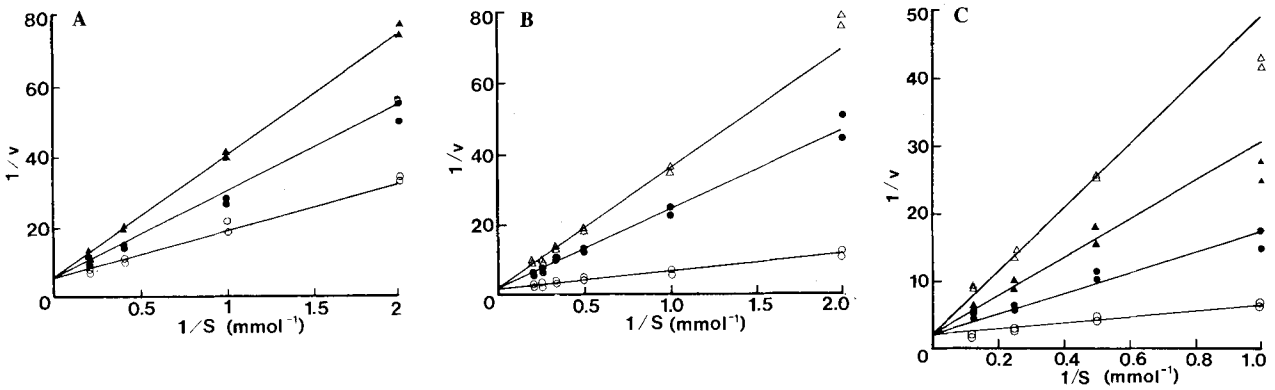

Fig. 2 (A) Competitive inhibition of chitin synthetase activity by polyoxin B. Resuspended high-speed particulate fractions were prepared as described and aliquots containing 85 μg protein added to reaction mixtures containing 12 m*M* GlcNAc and the indicated concentrations of UDPGlcNAc. The following polyoxin B concentrations were used: ○-○, 0 μM; ●-●, 25 μM; ▲-▲, 50 μM. The specific activity of UDP-^{14}C-GlcNAc was 2.4 × 10^8 dpm/mmol. Reaction mixtures were incubated for 0, 5, and 10 min at 30°C and the amount of chitin synthesized determined. Velocity (nmol product/min) was calculated and data plotted in the double reciprocal form. (B) Competitive inhibition of chitin synthetase activity by UDP. Experimental details are as above, except that the high-speed particulate fraction used in each reaction mixture contained 95 μg protein. The concentrations of UDP were ○-○, 0 m*M*; ●-●, 3 m*M*; △-△, 5 m*M*. (C) Competitive inhibition of chitin synthetase activity by nikkomycin. Experimental details are as above, except that the high-speed particulate fractions used in each reaction mixture contained 150 μg protein, the specific activity of UDP-^{14}C-GlcNAc was 1.8 × 10^8 dpm/mmol, and the following concentrations of nikkomycin were used: ○-○, 0 μM; ●-●, 5 μM; ▲-▲, 10 μM, △-△, 20 μM.

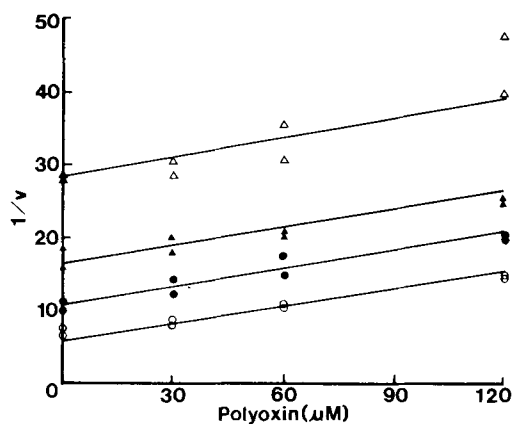

Fig. 3. Inhibition of chitin synthetase activity by UDP and polyoxin B. Resuspended high-speed particulate fractions were prepared as described and aliquots containing 150 μg of protein were added to reaction mixtures containing 1.2 m*M* UDPGlcNAc, 12.5 m*M* GlcNAc, and various combinations of UDP and polyoxin B. The specific activity of UDP-^{14}C-GlcNAc was 7.2 × 10^8 dpm/mmol. Reaction mixtures were incubated for 0, 5, and 10 min at 30°C and the amount of synthesized chitin determined. Velocities (nmol product/min) were calculated and data plotted by the method of Yonetani and Theorell [27]. Each point represents an individual determination. Polyoxin-B concentrations were as indicated and the following concentrations of UDP were used: ○-○, 0 m*M*; ●-●, 0.8 m*M*; ▲-▲, 1.6 m*M*; △-△, 3.2 m*M*.

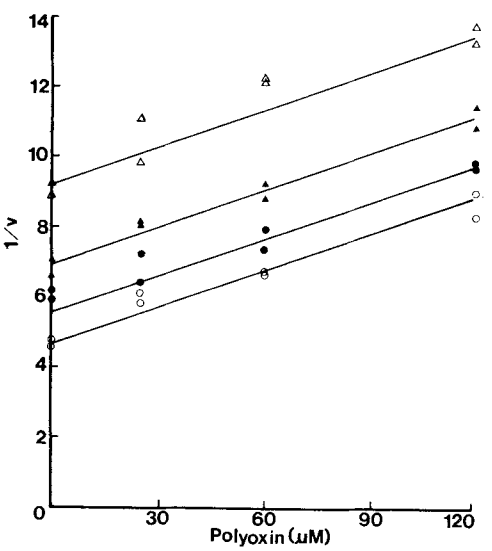

Fig. 4. Inhibition of chitin synthetase activity by nikkomycin and polyoxin B. Experimental details are as those in the legend of Fig. 3, except that nikkomycin was present in place of UDP. Polyoxin-B concentrations were as indicated and the following concentrations of nikkomycin were used: ○-○, 0 μM; ●-●, 1 μM; ▲-▲, 2 μM; △-△ 4 μM.

chitin synthetase from *Mucor rouxii* [22] and 2.0 m*M* for the enzyme from *Saccharomyces cerevisiae* [6]. The $K_{i\ app}$ polyoxin B (~ 32 μM is higher than that reported previously. Selitrennikoff and Zucker [25] found a $K_{i\ app}$ of 6 μM at pH 8.0 for a *slime* strain of *N. crassa* using Tris buffer. However, the difference between these two estimates may be due to the differences between strains and assay conditions in these reports. The effect of nikkomycin ($K_{i\ app}$ 2 μM) on chitin synthetase from *N. crassa* has not been previously tested. Digitonin-solubi-

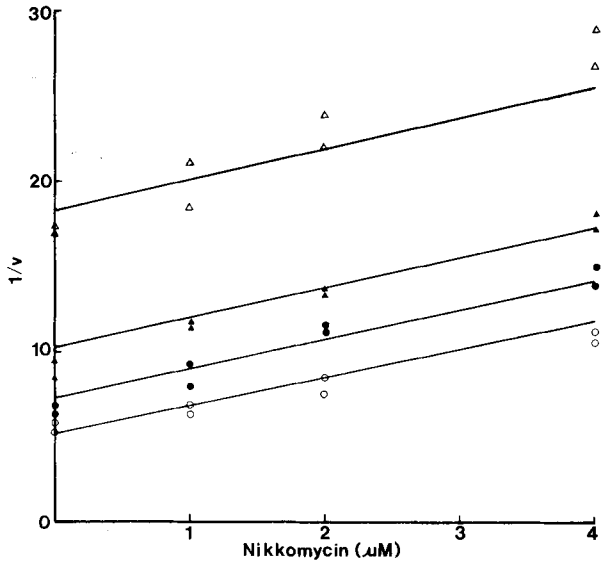

Fig. 5. Inhibition of chitin synthetase activity by nikkomycin and UDP. Experimental details are as those in the legend of Fig. 3, except that nikkomycin was present in place of polyoxin B. Nikkomycin concentrations were as indicated and the following concentrations of UDP were used: O-O, 0 m*M*; ●-●, 0.8 m*M*; ▲-▲, 1.6 m*M*; △-△, 3.2 m*M*.

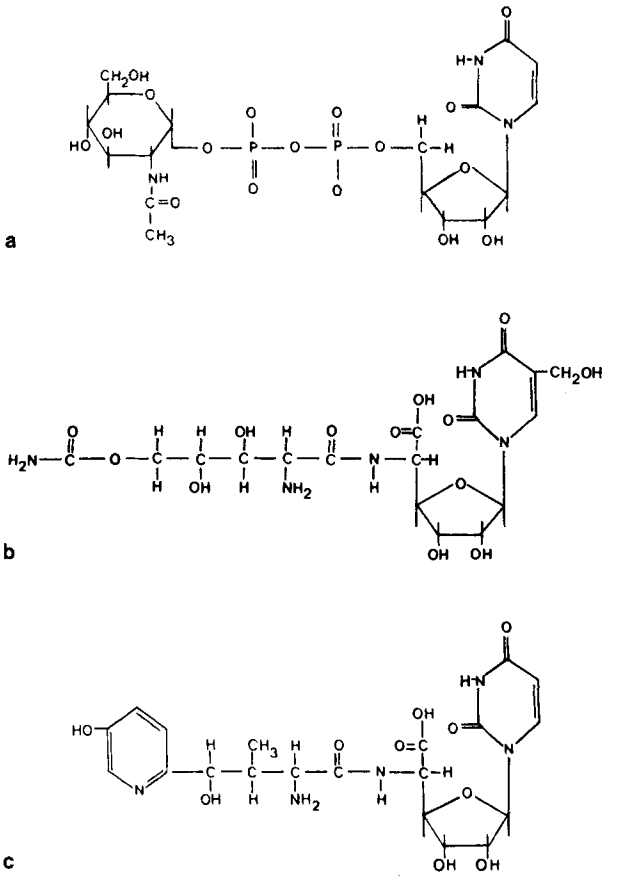

Fig. 6. Structural formulae of UDPGlcNAc (a), polyoxin B (b), and nikkomycin Z (c).

lized chitin synthetase from *Coprinus cinereus* had a $K_{i\ app}$ of 0.5 μM [2] while chitosomal preparations from *M. rouxii* [19] and *Agaricus bisporus* [12] gave values of 0.5 μM and 1.5 μM, respectively.

The second approach, using mixed-inhibitor experiments, enabled us to determine that the linear competitive inhibition by UDP, polyoxin B, and nikkomycin is due, in each case, to binding at the same enzyme site. The data presented here show a pattern of parallel lines for each pairwise combination of inhibitors (Figs. 3–5). The interaction coefficient, α, equals infinity (see *Materials and Methods*), indicating that each of these inhibitors completely excludes the others; in other words, the binding of each of the inhibitors is exclusive and they all bind at the same site. Taken together, these results support the conclusion that the three inhibitors *compete with each other and the substrate* for binding to chitin synthetase. Hori et al. [13–15] have compared the binding of UDPGlcNAc and polyoxins to chitin synthetase and have concluded that there is a specific binding site on the enzyme for the uridine moiety of UDPGlcNAc. Our results support this view (see Fig. 6 for structures of UDPGlcNAc, polyoxin B, and nikkomycin). Polyoxin B, UDP, and nikkomycin each possess a pyrimidine-nucleoside moiety. We have shown that GlcNAcIP (the sugar-phosphate component of the substrate) does not inhibit chitin synthetase, providing further evidence that the pyrimidine moiety is important for binding. In addition, Hori et al. [13] report that the carbamoyl polyoxamic acid moiety of the polyoxins is involved in stabilizing the enzyme–inhibitor complex. The stabilizing influence of this end group is likely to be responsible for the extreme potency (i.e., very low $K_{i\ app}$) of the polyoxins and nikkomycins. The observation that UDP does not possess such a group and has a much larger $K_{i\ app}$ lends further support to this idea.

As far as we are aware, mixed-inhibitor experiments have not been previously utilized to explore structure–function relationships of either antibacterial or antifungal antibiotics. We feel that this type of experiment will provide extremely valuable information for the identification of structures that are critical for enzyme inhibition.

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