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Synthesis and Biological Activity of Tripeptidyl Polyoxins as Antifungal Agents

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Three tripeptidyl polyoxins were synthesized and found to inhibit *Candida albicans*. Compared with the naturally occurring polyoxin D, the three synthetic polyoxins had little effect on chitin synthetase when assayed with a *C. albicans* membrane preparation. However, all the compounds inhibited growth, affected cell morphology in a manner similar to that of polyoxin D, and were hydrolyzed by cell extracts of *C. albicans*. Hydrolysis did not occur extracellularly, and at least one of the synthetic polyoxins, leucyl-norleucyl-uracil polyoxin C, inhibited peptide uptake, suggesting entrance into the cell via the peptide transport system. Thus, the intact tripeptidyl polyoxins are inactive prodrugs that are converted to active moieties by cellular enzymes.

The application of peptides as carriers for toxic agents into cells has been discussed extensively in recent years (13). A variety of microorganisms are known to have peptide transport systems which translocate di- and oligopeptides against a concentration gradient (2, 12, 14). Thus, peptides acting as carriers can deliver toxic agents into the cell, leading to high intracellular concentrations which ultimately cause cell death. Ideally, the toxic agent should be specific for the target cell or at least have a much higher affinity for the pathogen than for the host cells.

Candida albicans is a medically important fungus and a primary cause of death for patients with weakened immune systems (7). As with other fungi, chitin is an important structural component of the cell wall of *C. albicans*. The synthesis of this polysaccharide is catalyzed by chitin synthetase, a membrane-bound enzyme which is inhibited in phytopathogenic fungi by a family of peptidyl-nucleoside antibiotics called polyoxins (6). Recently, we showed that chitin synthetase from *C. albicans* is strongly inhibited by polyoxin D and a series of dipeptidyl polyoxin analogs, and that these peptidyl antibiotics at millimolar concentrations cause death of this yeast (1; P. Shenbagamurthi, H. Smith, J. M. Becker, A. S. Steinfeld, and F. Naider, J. Med. Chem., in press).

Clearly the high concentration of polyoxin D required to kill the organism minimizes the therapeutic value of this family of drugs for *C. albicans*. Our studies showed, however, that the peptide portion of polyoxin can be structurally altered without loss of activity against chitin synthetase (Shenbagamurthi et al., in press). Taking advantage of this observation, we have synthesized a new group of polyoxin analogs designed to permeate *C. albicans* more thoroughly.

The naturally occurring polyoxins have the structure depicted in Fig. 1 and are either dipeptidyl or tripeptidyl nucleosides. Although there is evidence suggesting that the polyoxins enter fungal cells via a peptide transport system (3, 11), preliminary studies showed that polyoxin D did not compete with the uptake of radiolabeled trimethionine (Met_3) into *C. albicans* (Shenbagamurthi et al., in press). Similarly, a series of dipeptidyl polyoxins that we previously investigated did not significantly inhibit Met_3 uptake (Shenbagamurthi et al., in press). Since the polyoxins can be considered peptides with unusual side chains, we reasoned that a tripeptidyl polyoxin derivative made up of commonly found amino acid residues would have a greater chance for recognition by the peptide transport system. These peptidyl polyoxins must have a free C_2'' amine group and a free carboxyl moiety in the vicinity of the C_5' position since previous studies have established these requirements for activity (8, 9, 14). Investigations of the peptide transport system in *C. albicans* established that peptides containing hydrophobic amino acid residues have a higher affinity for the transport system than peptides made up of more hydrophilic components (2, 10). Our strategy included modifications which should improve recognition by the peptide transport system in *C. albicans*.

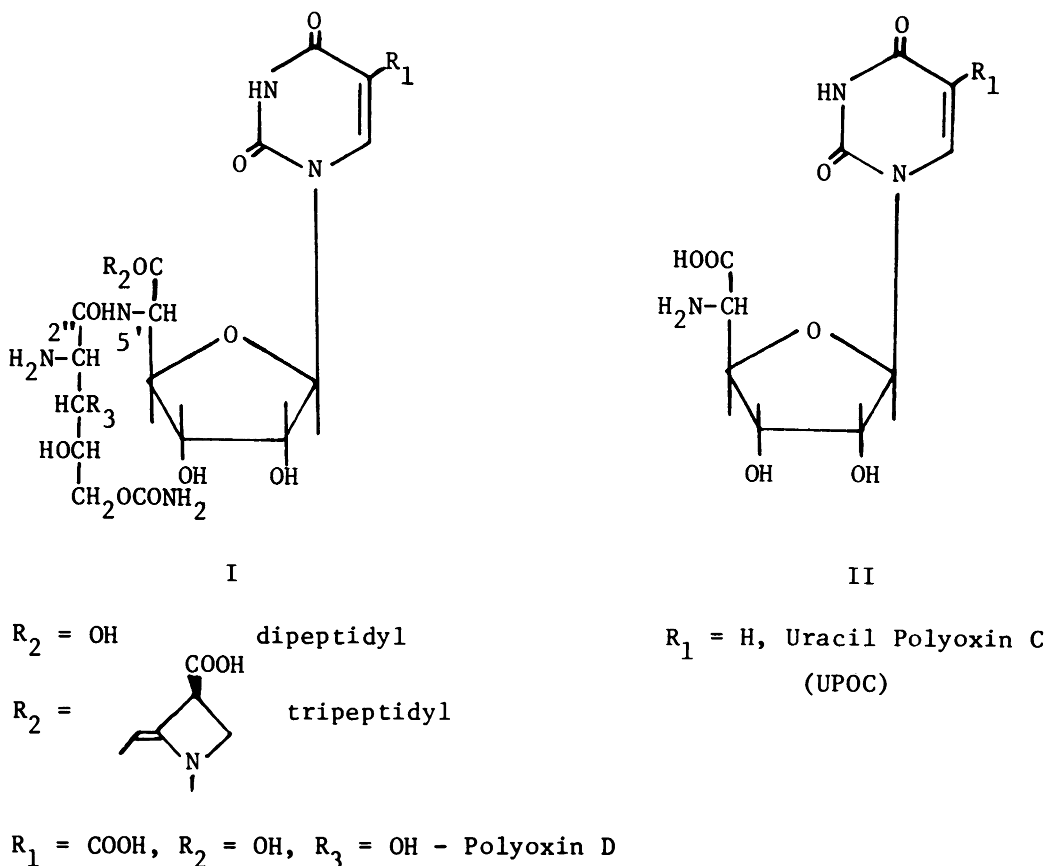

FIG. 1. Structural formulae of dipeptidyl and tripeptidyl polyoxins.

This paper presents the synthesis and biological properties of tripeptidyl polyoxins designed on the basis of the structural specificities of both the peptide transport system and chitin synthetase of *C. albicans*.

MATERIALS AND METHODS

Abbreviations. Abbreviations used in this paper are as follows: BuOH, butanol; DMF, dimethylformamide; Gly, glycine; HOAc, acetic acid; HPLC, high-pressure liquid chromatography; Leu, L-leucine; MEC, minimal effective concentration; Met, methionine; Met_3, trimethionine; Nle, L-norleucine; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; OBzl, benzyl ester; ONp, nitrophenyl ester; TFA, trifluoroacetic acid; Z, carbobenzoxy; UPOC, uracil polyoxin C. The tripeptidyl polyoxins are named as derivatives of polyoxin D or UPOC (Fig. 1).

Synthesis of Leu-polyoxin D. To a solution of Z-Leu-ONp (46 mg, 0.12 mmol) and polyoxin D · TFA (63.5 mg, 0.1 mmol) in a mixture of DMF (1 ml) and water (0.3 ml), NMM (0.033 ml, 0.3 mmol) was added, and the mixture was stirred at room temperature for 18 h. The reaction mixture was acidified with HOAc (0.1 ml), the solvents were evaporated in vacuo, and the residue was precipitated by adding ether. The crude Z-Leu-polyoxin D obtained after filtration was subjected to transfer hydrogenation without purification.

To a solution of crude Z-Leu-polyoxin D in methanol (2 ml), palladium black (ca. 50 mg) was added. The mixture was stirred and treated with 90% HCOOH (0.2 ml). After 30 min, the catalyst was removed by filtration through celite, and the filtrate was evaporated to dryness. The residue was dissolved in 2 ml of water-TFA (99.975:0.025, vol/vol) and was injected onto a μBondapak C_{18} (1 in. [ca. 2.5 cm] Semiprep) column of a Waters prep LC/system 500, which had been equilibrated with the same solvent system. The column was eluted at a flow rate of 50 ml/min, the fractions were analyzed by analytical HPLC, and those corresponding to the main peak were pooled and evaporated in vacuo at room temperature. The residue was dissolved in water (5 ml) and filtered through a microfilter (0.45 μm), and the filtrate was freeze-dried. Yield, 33 mg (44.1% based on polyoxin D) $[\alpha]_D^{25}$ = +16.67 (c 0.12, H_2O); R_f(n-BuOH–HOAc–H_2O, 4:1:2) = 0.08. NMR (Me_2SO-d_6) δ 0.89 (d, J = 5.7 Hz, 6, side chain CH_3), 1.45 to 1.75 (m, 3, CH_2 and CH), 4.51 (m, 1, Leu α-C*H*), 4.61 (m, 1, C_5, H), 5.81 (d, J = 4.4 Hz, 1, C_1'H), 8.09 (d, J = 8.4 Hz, 1, N*H*), 8.37 (s, 1, C_6H), 8.60 (d, J = 8 Hz, 1, N*H*). For C, H, N analysis, the freeze-dried sample was further dried by heating in vacuum over P_2O_5 at a temperature of 60°C for 24 h. Analysis ($C_{17}H_{23}N_5$ O_{18} · $2H_2O$ · 1.7 TFA) C, H, N.

Synthesis of Leu-Nle-UPOC. To a solution of Z-Nle-

ONp (278 mg, 0.72 mmol) and UPOC · HCOOH (200 mg, 0.6 mmol) in a mixture of DMF (2 ml) and water (0.5 ml), NMM (0.13 ml, 1.2 mmol) was added, and the mixture was stirred at room temperature for 20 h. The reaction mixture was acidified with HOAc (0.2 ml), the solvents were evaporated in vacuo, and the residue was precipitated by adding ether. The solid was filtered, dissolved in DMF (0.3 ml), and reprecipitated by adding 2% aqueous HOAc. The precipitate was filtered, washed with water, and dried. The yield of Z-Nle-UPOC was 168 mg (51%); melting point, 255 to 256°C (d); R_f (n-BuOH–HOAc–H_2O, 4:1:2) = 0.74.

Z-Nle-UPOC (150 mg) was hydrogenolized by using palladium black and formic acid as described for the preparation of Leu-polyoxin D. The yield of Nle-UPOC · HCOOH was 90 mg (74.3%). $[\alpha]_D^{25}$ = +49.33 (c 0.15, H_2O); R_f (n-BuOH–HOAc–H_2O, 4:1:2) = 0.36. This product (60 mg, 0.135 mmol) was coupled with Z-Leu-ONp (62 mg, 0.16 mmol) in the presence of NMM (0.03 ml, 0.27 mmol) in DMF (1 ml) and water (0.3 ml). After a reaction period of 20 h, the product Z-Leu-Nle-UPOC was isolated as described above. Yield, 62 mg (69.3%); melting point, 226 to 228°C (d); R_f (n-BuOH–HOAc–H_2O, 4:1:2) = 0.73. A 50-mg portion of Z-Leu-Nle-UPOC was subjected to catalytic transfer hydrogenation as described above to yield 27 mg (62.2%) of Leu-Nle-UPOC · HCOOH. The product was 98% homogenous as judged by analytical HPLC on a μBondapak C_{18} column with methanol-water-TFA (400:600:0.25, vol/vol/vol) as the eluent. $[\alpha]_D^{25}$ = +18.57 (c 0.14, H_2O); R_f (*n*-BuOH–HOAc–H_2O, 4:1:2) = 0.43. NMR (Me_2SO-d_6) δ 0.87 (m, 9, side chain CH_3), 1.1 to 1.9 (m, 9, CH_2 and CH), 5.6 (d, J = 8.0 Hz, 1, C_5H), 5.78 (d, J = 6.0 Hz, 1, $C_1'H$), 7.68 (d, J = 8.0 Hz, 1, C_6H), 7.94 (d, J = 7.8 Hz, 1, amide N*H*), 8.45 (d, 1, amide N*H*). Analysis ($C_{22}H_{35}O_9N_5 \cdot 2H_2O$) C, H, N.

Synthesis of Nle-UPOC-Leu. 1-(5′-azido-2′,3′-*O*-cyclohexylidine-5′-deoxy-β-D-allofuranuronosyl)-uracil (I). A solution of 1-(5′-azido-5′-deoxy-β-D-allofuranuronosyl)-uracil (250 mg, 0.8 mmol), 1:1-diethoxycyclohexane (206 mg, 1.2 mmol), and 70% $HClO_4$ (0.004 ml) in DMF (1.6 ml) was heated in a round-bottomed flask at 70°C at 15 mm of pressure. The reaction vessel was fitted with a reflux condenser of such a length that DMF refluxed, but ethanol, the by-product of the reaction, was distilled off. After 2 h, the reaction mixture was neutralized with triethylamine (0.2 ml) and evaporated to dryness in vacuo. The residue was acidified with HOAc (0.2 ml) and partitioned between saturated NaCl and CH_2Cl_2. The CH_2Cl_2 layer was dried over magnesium sulfate, filtered, and concentrated to a syrup. Crystallization from CH_2Cl_2-hexane gave 1-(5′-azido-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl)-uracil. Yield, 273 mg (87%); melting point, 155 to 157°C.

1-(5′-Azido-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl-Leu OBzl)uracil (II). A solution of 1-(5′-azido-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl)-uracil (236 mg, 0.6 mmol), Leu-OBzl · TsOH (248 mg, 0.63 mmol), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (156 mg, 0.63 mmol), and NMM (0.069 ml, 0.63 mmol) in DMF (1 ml) was stirred at room temperature overnight. After removal of the solvent in vacuo, the residue was taken up in ethyl acetate, washed three times each with 5% citric acid and water, and dried. It was then concentrated and crystallized from ethyl acetate-hexane. Yield, 290 mg (81%); melting point, 143 to 145°C.

1-(5′-Amino-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl-Leu)uracil · HCOOH (III). The azido compound (II) (284 mg) was subjected to catalytic transfer hydrogenation with palladium black-formic acid to yield 245 mg (98%) of 1-(5′-amino-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl-Leu)uracil · HCOOH. Melting point, 192 to 195°C (d).

1-[5′-(*N*-Z-Nle)-amino-2′,3′-*O*-cyclohexylidene-5′-deoxy-β-D-allofuranuronosyl Leucine]uracil (IV). The amino compound (III) (237 mg, 0.45 mmol) was dissolved in DMF (1.5 ml), and Z-Nle-ONp (208 mg, 0.54 mmol) and 1-hydroxybenzotriazole (69 mg, 0.45 mmol) were added to the solution, followed by NMM (0.1 ml, 0.9 mmol). After 2 h, the reaction mixture was diluted with water (15 ml) and acidified with 5% citric acid. The crude tripeptide (IV) was isolated and purified by precipitation from DMF-ether. Yield, 260 mg (79.5%); melting point, 218 to 220°C.

1-[5′(Nle)-amino-5′-deoxy-β-D-allofuranuronosyl-Leu]-uracil Nle-UPOC-Leu. Compound IV (240 mg, 0.33 mmol) was dissolved in 80% HOAc (5 ml), and the solution was held at 100°C for 6 h. The mixture was evaporated to dryness, and the residue was taken up in DMF and precipitated with ether to give crude Z-Nle-UPOC-Leu (144 mg, 67.6%). The crude Z-Nle-UPOC-Leu (120 mg, 0.185 mmol) was subjected to catalytic transfer hydrogenation as described earlier to give Nle-UPOC-Leu · HCOOH (94 mg, 91%). The crude material (25 mg) was subjected to purification on Semiprep HPLC with methanol-water-TFA (400:600:0.25, vol/vol/vol) used as the eluent to give 17 mg of pure Nle-UPOC-Leu · TFA. $[\alpha]_D^{25}$ = −11.72 (c 0.13, H_2O); R_f (*n*-BuOH–HOAc–H_2O, 4:1:2) = 0.47. NMR (Me_2SO-d_6) δ 0.84 (m, 9, side chain CH_3), 1.15 to 1.65 (m, 9, CH_2 and CH), 4.76 (m, 1, $C_5'H$), 5.6 (d, J = 8.1 Hz, 1, C_5H), 5.82 (d, J = 7.5 Hz, 1, $C_1'H$), 7.77 (d, J = 8.1 Hz, 1, C_6H), 8.35 (d, J = 8.2 Hz, 1, amide N*H*), 8.82 (d, J = 7.9 Hz, 1, amide N*H*). Analysis, ($C_{22}H_{35}O_9N_5 \cdot 2H_2O \cdot 0.7$ TFA) C, H, N.

Organism and growth conditions. *C. albicans* H-317, a clinical isolate from the Centers for Disease Control, Atlanta, Ga., was used in all experiments. The yeast was maintained on slants of YEPD agar containing 1% yeast extract, 2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 2% glucose, and 2% agar (Difco) (all percentages wt/vol). The organism was transferred monthly to a fresh agar slant, incubated for 24 h at 37°C, and stored at 4°C. The mineral growth medium used for all experiments was yeast nitrogen base (Difco) without amino acids and $(NH_4)_2SO_4$. The medium was filter sterilized, and sterile solutions of glucose as carbon and energy sources and isoleucine as nitrogen source were added aseptically to bring the medium to 2% (wt/vol) glucose and 0.5% (wt/vol) isoleucine. For growth, cells were taken from a YEPD agar slant and inoculated to give 10^4 cells per ml in 25 ml of fresh medium contained in a 125-ml flask. The culture was aerated by stirring with a magnetic stir bar and was grown for 18 to 24 h at 37°C. A portion (7 ml) of this culture was inoculated into 93 ml of fresh medium (giving 10^4 cells per ml) in a 500-ml flask fitted with a side arm. The culture was stirred vigorously at 37°C. Growth was determined turbidimetrically at 400 to 420 nm (blue filter) with a Klett-Summerson photoelectric colorimeter. One Klett unit corresponded to

5×10^4 cells per ml. Under these growth conditions, the cells remained in the yeast phase.

Chitin synthetase assay. Chitin synthetase activity obtained in a mixed membrane fraction (4) from *C. albicans* H-317 was assayed in the presence and absence of polyoxin compounds by measuring the incorporation of *N*-acetylglucosamine into chitin. For the assay of total chitin synthetase activity, the enzyme was activated with trypsin in a reaction assay containing 125 μl of the membrane fraction (5.17 mg of protein per ml, 25 μl of 4 mM ATP, 15 μl of 0.5 M imidazole buffer [pH 6.5], 5 μl of trypsin at 2 mg/ml, 35 μl of distilled water). After incubation at 37°C for 10 min, 5 μl of trypsin soybean inhibitor (3 mg/ml), 10 μl of 0.8 M *N*-acetylglucosamine, 5 μl of 50 mM UDP-[^{14}C]*N*-acetylglucosamine (200,000 cpm/mol), and 25 μl of water for the control of polyoxin analogs at 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6} M were added for each assay. Samples of 50 μl were removed after 5, 20, 40, and 60 min and added to 20 μl of glacial acetic acid; 2 ml of cold 60% ethanol was then added. The labeled chitin precipitate was then separated by filtration on a Whatman 934-AH filter prewashed with 20 mM sodium pyrophosphate and then washed twice with 10 ml of an acetic acid-ethanol solution. The filters were placed in scintillation cocktail, and the label was counted by liquid scintillation for the incorporation of radioactivity into chitin.

Preparation of cell extract. Cells of *C. albicans* H-317 were harvested at the late logarithmic phase of growth and were washed twice with distilled water. For each gram of yeast cells (wet weight), 1.5 to 2.0 ml of 0.1 M NaCl, 0.1 M Tris-hydrochloride (pH 7.2), and 5.0 g of glass beads (Minnesota Mining & Manufacturing Co., St. Paul, Minn., diameter, 50 to 70 μm) were added to a 50-ml Braun homogenizer vessel, cooled with CO_2, and homogenized for 1.5 to 2 min. The glass beads, cell debris, and unbroken cells were removed by centrifugation (25,400 × *g* for 30 min). The crude extract was then dialyzed against 100 volumes of 0.1 M NaCl–0.1 M Tris-hydrochloride (pH 7.2) for 24 h with a buffer change after 3 h. After the addition of glycerol to a final concentration of 20%, the extract was then stored at −20°C and used as such in the peptidase assays.

Peptidase assay. A portion of a cell extract (0.2 mg of protein per ml) from *C. albicans* H-317 (usually one-half to one-fourth of the assay volume) was incubated at 37°C with the various polyoxin compounds (10 mM in distilled sterile water). At intervals of 30 and 60 min, 10 μl were removed from the reaction mixture and applied to 3MM Whatman filter paper for subsequent electrophoresis.

Electrophoresis. Electrophoresis was carried out in a model LT-36 electrophoresis tank with E.C. 123 coolant and an HV-5000 power supply (Savant Instruments, Inc., Hicksville, N.Y.). Pyridine acetate buffer, pH 3.5, was prepared from glacial acetic acid-pyridine-water (10:1:89). Samples were applied to 3MM Whatman paper and run at a gradient of 50 V/cm for 2 h. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5%, wt/vol) in 95% aqueous acetone, and developed by heating in a ventilated oven.

Determination of MEC, growth inhibition, and viability. *C. albicans* H-317 was grown at 37°C with stirring in yeast nitrogen base (Difco) containing 0.5% isoleucine and 5.0% glucose. Cells were harvested by centrifugation at the early logarithmic phase of growth (approximately 60 Klett units; blue filter, Klett-Summerson photoelectric colorimeter). The cells were washed with sterile yeast nitrogen base and resuspended to 3×10^6 cells per ml for use as the inoculum for microtiter plates (Corning Glass Works, Corning, N.Y.). Fifty microliters of yeast nitrogen base was added to microtiter wells, and 100 μl of various polyoxin analogs or polyoxin D in yeast nitrogen base was added to the first well; the concentration of the analog was adjusted by twofold serial dilutions. To each well was then added 25 μl of inoculum. With this procedure, each well contained various concentrations of the drug and 10^6 cells per ml in a total volume of 75 μl. The microtiter plates were incubated at 37°C in a humid chamber. After 48 h of incubation, 50 μl was removed from each well and examined microscopically. The MEC is defined as the lowest concentration of drug which resulted in some (5%) morphologically abnormal cells at 48 h. The number of potential viable units was determined by hemacytometer counting, and the percentage of growth inhibition was calculated by comparison of the number of cells at 48 h in the control well (no treatment) with the number of cells in drug-treated wells. A portion was removed from each well after 48 h of incubation and was serially diluted through yeast nitrogen base. A sample from the dilution calculated to have between 50 and 200 cells was spread on potato dextrose agar (Difco) and incubated for 2 days at 37°C. Viable colonies were counted directly. The percentage of viability was calculated by comparing the number of viable colonies with the number of potential viable cells by direct counting.

Transport studies. Cells were grown for 6 to 8 h (40 to 70 Klett units; 2×10^4 to 3.5×10^6 cells per ml) and were then harvested by filtering through a membrane filter (pore size, 0.65 μm), washed twice with cold (0 to 4°C) distilled water, resuspended so that a 1:10 dilution contained 1.4 mg (dry weight) per ml (30 Klett units), and kept cold until assayed. When assayed for uptake of peptides, the cells were incubated at 37°C for 10 min and added to an equal volume (0.5 ml) of reaction mixture containing 2% (wt/vol) glucose, 0.03 M citric acid-KH_2PO_4 buffer (pH 3.5), and radioactive trimethionine (3×10^{-5} M, 1.0 μCi/μmol) with or without polyoxin analogs at a 10-fold concentration of trimethionine. At intervals, samples of the reaction mixture were applied to filters (pore size, 0.45 μm) and were washed twice with 2.0 ml of distilled water. The filters were placed in 5 ml of Bray solution and were counted in a Searle Isocap/300 6868 liquid scintillation system. There was no peptide adsorption to the cell surface or sticking to the filter since at 0°C the counts were at background levels. The uptake results, calculated on the basis of 80% counting efficiency and the known specific activity of the peptide (1.0 μCi/μmol), are expressed as nanomoles of peptide taken up per milligram (dry weight) of cells.

RESULTS

Chemical synthesis. Preparation of Leu-polyoxin D and Leu-Nle-UPOC was accomplished by adding Z-Leu-ONp to the unprotected dipeptidyl nucleoside in a DMF-water mixture (Fig. 2). The crude products were then deprotected by

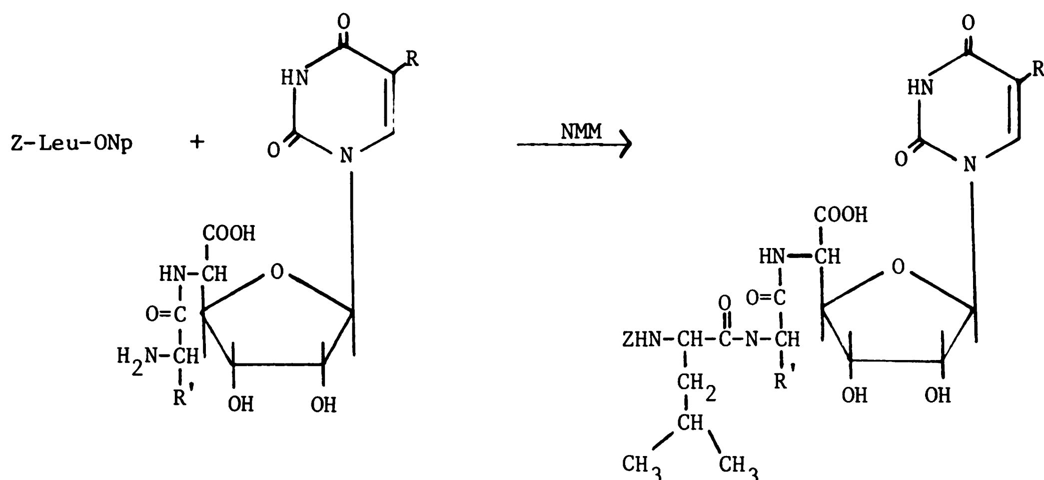

FIG. 2. Structural formulae of tripeptidyl polyoxins and schematic representation of their synthesis.

using transfer hydrogenation with palladium black and formic acid. In the case of Leu-Nle-UPOC and Leu-polyoxin D, the overall yield of the coupling and deprotection steps was approximately 45%. Both products were 98% pure as judged by HPLC on a reversed-phase column.

The synthesis of Nle-UPOC-Leu was first attempted by coupling Z-Nle-UPOC with Leu-OBzl, using dicyclohexylcarbodiimide/1-hydroxybenzotriazole for carboxyl activation. Thin-layer chromatographic analysis of this reaction revealed that one major product was not obtained. Similar results were found by using the mixed anhydride reaction. The side reactions occurring during these syntheses could have resulted either from the ribose hydroxyl groups (2′,3′) or from the participation of tautomeric species of the uracil ring. To minimize reaction with the 2′ or 3′ OH groups, 1-(5′-azido-2′,3′-*O*-cyclohexylidene-5′-deoxy-D-allofuranuronosyl-uracil) was prepared (5) and reacted with Leu-OBzl. Side reactions were still observed with dicyclohexylcarbodiimide activation, and the reaction was incomplete after 18 h at room temperature. When isobutylchloroformate was used as the carboxyl activating group, the amount of side product was dependent on the time of activation. Short activation times (<3 min) resulted in the formation of one major product as judged by thin-layer chromatography and analytical HPLC, whereas activation times of >5 min resulted in the formation of two products in approximately equal amounts.

To minimize the lifetime of the activated carboxyl moiety, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was used to slowly generate the mixed anhydride in the presence of Leu-OBzl. No side product was observed, and the product was obtained in 81% yield. This product was reduced to the corresponding free peptidyl nucleoside by using palladium black-HCOOH in 98% yield. Z-Nle-ONp was then added by using 1-hydroxybenzotriazole acceleration to give a protected tripeptidyl polyoxin in 80% yield. Final deprotection was accomplished by acidolysis in 80% acetic acid followed by catalytic transfer hydrogenation. The crude product thus obtained was purified by semipreparative HPLC on a reversed-phase column to give homogenous Nle-UPOC-Leu.

The final products exhibited the expected NMR resonances and had the calculated elemental analyses. Interestingly, despite having identical molecular formulas, Leu-Nle-UPOC and Nle-UPOC-Leu separated on reversed-phase HPLC (unpublished data). We conclude that positional isomerism affects mobility on reversed-phase columns and that the individual residues of a peptide interact differently with the support. Thus, it is not possible to predict mobility based solely on the overall hydrophobicity of the peptidyl-nucleoside conjugate.

Inhibition of chitin synthetase by tripeptidyl polyoxins. Activity of chitin synthetase was assayed by using a mixed membrane fraction from *C. albicans* H-317. Dose-response curves were plotted for the tripeptidyl-polyoxins (Fig. 3). Leu-polyoxin D caused negligible inhibition of chitin synthetase at concentrations up to 10^{-4} M, whereas Leu-Nle-UPOC and Nle-UPOC-Leu showed about a 40% inhibition at 10^{-4} M. Comparison of these results with the 50% inhibition dose values (concentration yielding 50% inhibition of activity) for polyoxin D and Nle-UPOC suggests that acylation of the C_2''-amino group with an amino acid resulted in marked decreases in activity. This is consistent with the requirement of a free C_2''-amino group for high

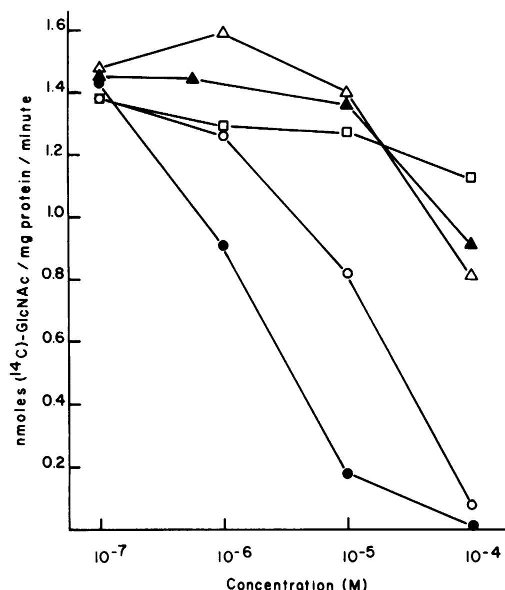

FIG. 3. Effect of synthetic polyoxins on chitin synthetase activity. The initial rate of enzyme activity was determined and plotted against the concentration of compound. Symbols: □, Leu-polyoxin D; ▲, Leu-Nle-UPOC; △, Nle-UPOC-Leu; ○, Nle-UPOC; ●, polyoxin D.

activity against chitin synthetase (8, 9, 13).

Effect of tripeptidyl polyoxins on cell morphology and growth. In a previous study, we found that polyoxin D caused severe alterations in the morphology of *C. albicans* (1). Therefore, we evaluated the tripeptidyl polyoxins to determine their MECs, that is, the lowest concentration at which some (5%) morphologically abnormal cells were observed under the microscope (Fig. 4, Table 1). Several analogs caused marked chaining of *C. albicans* (Fig. 4C and D). Others only caused cells to swell to two to three times their normal size (Fig. 4E and F). Polyoxin D, Leu-polyoxin D, and Nle-UPOC had MECs of 0.25 mM or less. Leu-Nle-UPOC had an MEC of 2 mM, and Nle-UPOC-Leu was slightly active at 4 mM (Table 1, Fig. 4). In addition to their effects on cell shape and size, several of the tripeptidyl polyoxins also inhibited the growth of the yeast (Table 1).

The effect of each drug on cell viability was determined by the number of colonies formed after incubation of a known number of cells with various polyoxin derivatives (Table 1). Polyoxin D and Leu-polyoxin D killed 50% of the cells at concentrations of 0.25 and 2 mM, respectively. None of the other compounds except Nle-UPOC killed the yeast at concentrations up to 4 mM.

Degradation of tripeptidyl polyoxins by whole cells and cell extracts of *C. albicans*. The ability of polyoxins to affect the growth and morphology of *C. albicans* is related to the inhibition of chitin synthetase by these antibiotics. Since both Leu-polyoxin D and Leu-Nle-UPOC had only poor activity against chitin synthetase (Fig. 3), it was somewhat surprising that they were effective against yeast cells in culture. To determine whether these compounds were converted to polyoxin D or Nle-UPOC by extracellular hydrolysis, we incubated them with whole cells. The cells were then centrifuged, and the supernatant was chromatographed by using high-voltage electrophoresis. No evidence of degradation or any change in the starting compounds was observed (data not shown). However, when a cell extract obtained by breaking the cells with glass beads was incubated with the same compounds, high-voltage electrophoresis revealed extensive degradation of the substrates (Fig. 5). Under our assay conditions (see above), Leu-Nle-UPOC was cleaved by cell extract to Nle, Leu, and UPOC with Nle-UPOC as an intermediate in the hydrolysis (data not shown), whereas Leu-polyoxin D was hydrolyzed to Leu and polyoxin D. Neither polyoxin D nor UPOC was degraded further. Interestingly, Nle-UPOC-Leu was partially hydrolyzed to Nle and UPOC-Leu. These results suggest that Leu-polyoxin D and Leu-Nle-UPOC enter the cell and are then converted to active species, probably polyoxin D and Nle-UPOC, respectively, which then inhibit chitin synthetase, causing morphological alterations and growth inhibition. In contrast, if Nle-UPOC-Leu did enter the cell, it would be converted to Nle and UPOC-Leu. These products are not expected to inhibit chitin synthetase significantly.

Interaction between peptides and tripeptidyl polyoxins. When 1% tryptone was incorporated into the culture medium, the morphological and toxic effects of each polyoxin were prevented. Since tryptone contains a variety of peptides, it was reasonable to conclude that these peptides prevented entry of the polyoxins, thereby blocking their inhibitory effects. To define further the ability of polyoxins to use the peptide transport system, the initial rate of uptake of radiolabeled Met_3 was measured in the presence of a 10-fold excess of these compounds (Fig. 6). Met_3 was previously found to enter *C. albicans* through the peptide transport system (10). Only Leu-Nle-UPOC significantly inhibited Met_3 transport, causing a 59% decrease in the initial rate of transport of this peptide. Gly-Met-Gly, a tripeptide known to be a competitive inhibitor of Met_3 uptake (10), is shown as a control.

DISCUSSION

In this communication, we report the successful synthesis of three tripeptidyl polyoxins

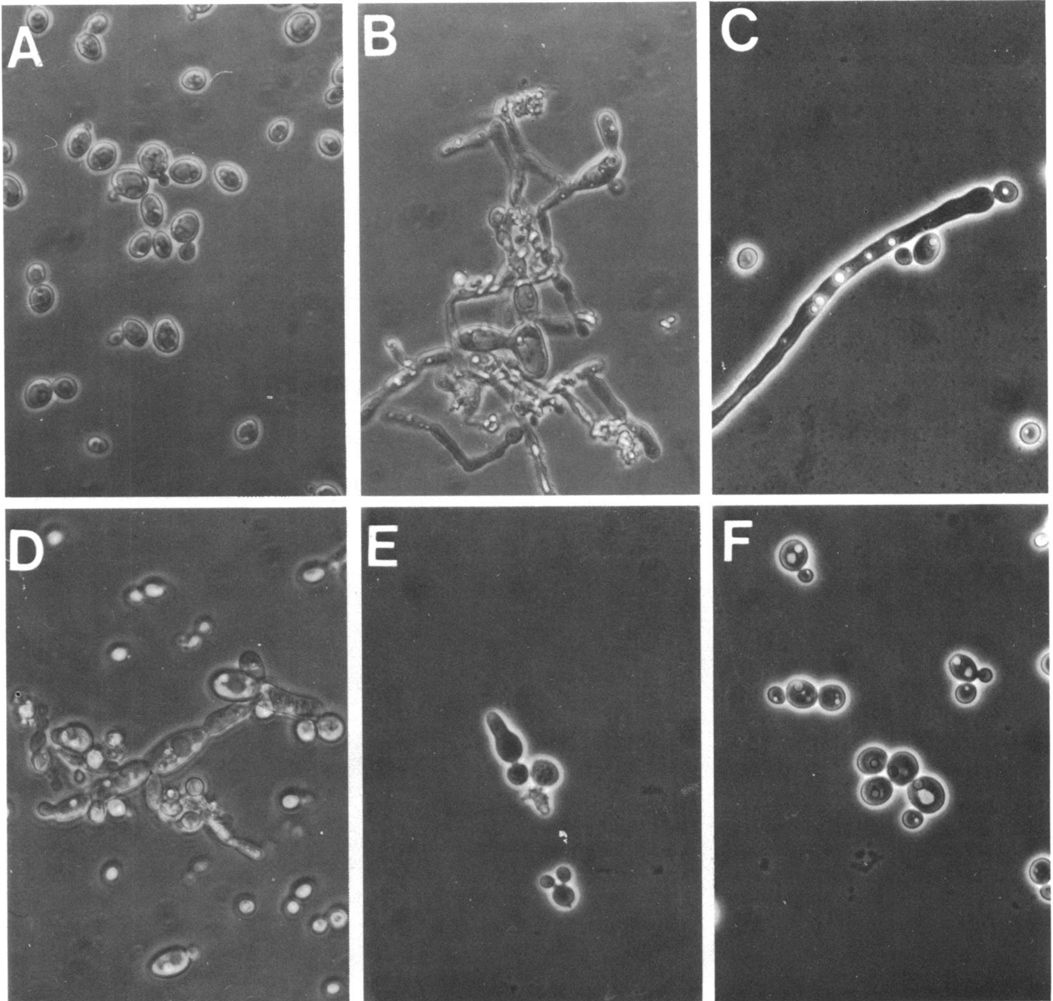

FIG. 4. Effects of compounds on cell morphology. *C. albicans* was incubated with various compounds for 48 h at 37°C and examined by phase-contrast microscopy (magnification, ×245). (A) Untreated control; (B) polyoxin D at 0.25 mM; (C) Leu-polyoxin D at 0.5 mM; (D) Nleu-UPOC at 0.25 mM; (E) Leu-Nleu-UPOC at 4 mM; (F) Nle-UPOC-Leu at 4 mM.

which have different biological activities against *C. albicans*. Previous studies have discussed the synthesis of compounds similar to Leu-polyoxin D and Leu-Nle-UPOC (9). However, to our knowledge this is the first report of the synthesis of a tripeptidyl polyoxin with an amino acid residue attached to the carboxyl position of uronic acid (Fig. 1, position R_2).

The tripeptidyl polyoxins are poor inhibitors of chitin synthetase from a mixed membrane fraction of *C. albicans* H317 (Table 1). Despite this fact, both Leu-polyoxin D and Leu-Nle-UPOC inhibit the growth of the yeast and cause morphological changes in a manner similar to polyoxin D and Nle-UPOC. In addition, at a concentration of 2 mM, Leu-polyoxin D kills 50% of the yeast cells. It is our belief that these compounds enter *C. albicans* and are converted to active agents inside the cell. We reach this conclusion based on the following observations. (i) Although Nle-UPOC-Leu was the most active analog against chitin synthetase in mixed membrane fractions (Fig. 3), it was the least active drug against intact cells (Table 1). (ii) None of the tripeptidyl polyoxins was hydrolyzed extracellularly. (iii) Leu-polyoxin D and Leu-Nle-UPOC were hydrolyzed to polyoxin D and Nle-UPOC (which was hydrolyzed further), respectively, by cell extracts, whereas Nle-UPOC-Leu was hydrolyzed to UPOC-Leu. This latter metabolite was expected to be inactive against *C. albicans*, whereas both polyoxin D

TABLE 1. Biological effects of polyoxin analogs

Compound	Chitin synthetase[a]	Transport inhibition (%)[b]	Hydrolysis[c]	Growth inhibition[d]	MEC (mM)[e]	Viability[f]
Leu-polyoxin D	$>10^{-4}$	11	+	0.25	0.25	2
Leu-Nle-UPOC	$>10^{-4}$	59	+	0.50	2	>4
Nle-UPOC-Leu	$>10^{-4}$	7	+	2	4	>4
Nle-UPOC	1.4×10^{-5}	5	+	0.12	0.12	1
Polyoxin D	1.8×10^{-6}	10	−	0.06	0.03	0.25
UPOC	$>10^{-4}$	3	−	4	>4	>4

[a] Molar concentration at which the enzyme is inhibited by 50%.

[b] The compound was added at 10-fold the peptide transport substrate. The value shown is the percent inhibition of the initial rate of transport.

[c] +, Compound was hydrolyzed by *C. albicans* cell extract. −, No hydrolysis by cell extract.

[d] Percent inhibition was calculated as 100 times the number of cells after 48 h of incubation with the compound divided by the number of cells in the control well (untreated). The value shown is the concentration (millimolar) of compound that gave 50% growth inhibition.

[e] The concentration of compound at which some morphological effects (about 5% of the cells) were visible microscopically.

[f] The value shown is the concentration (millimolar) of compound at which 50% of the cells were not viable.

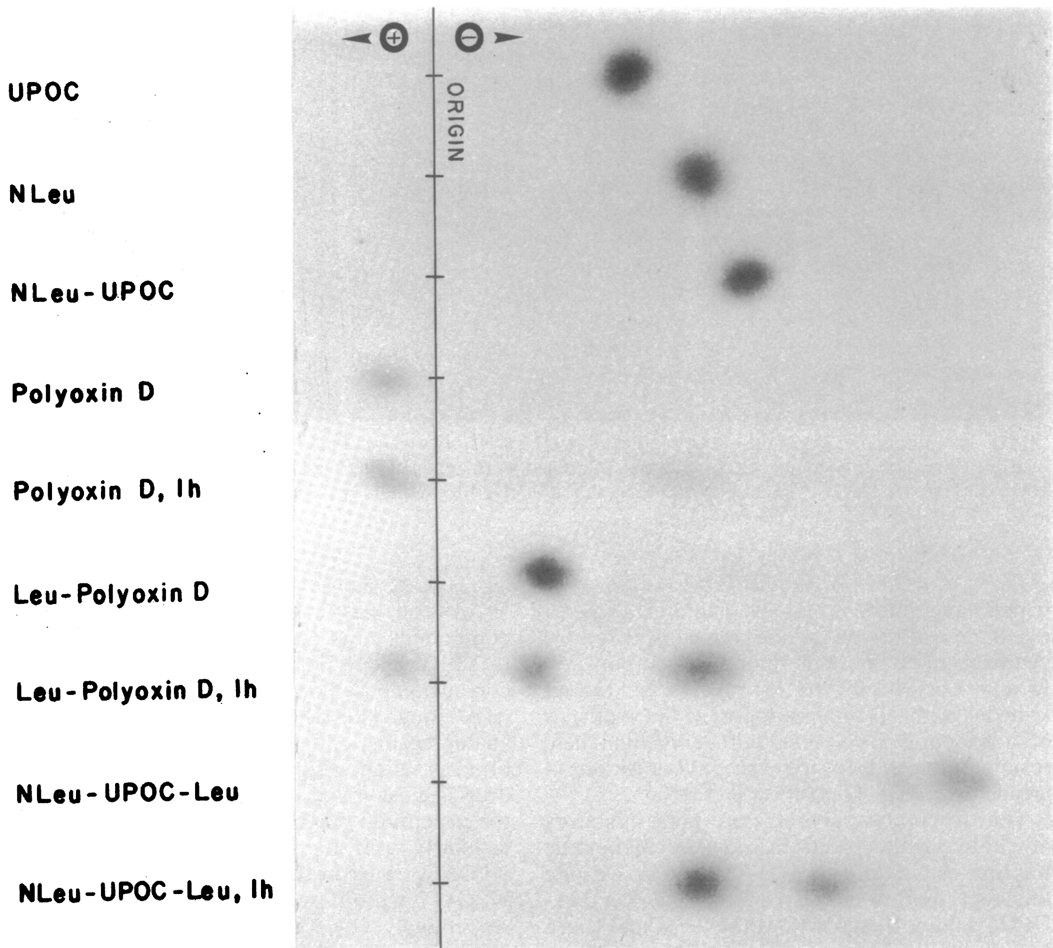

FIG. 5. High-voltage paper electrophoresis of resultant products from incubation of cell extract with Leu-polyoxin D and Nle-UPOC-Leu.

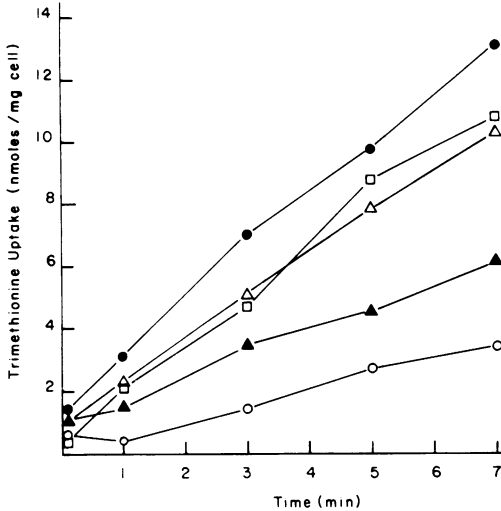

FIG. 6. Effects of various compounds on peptide uptake. Symbols: ●, control (uptake of Met_3); □, Leu-polyoxin D; △, Nle-UPOC-Leu; ▲, Leu-Nle-UPOC; ○, Gly-Met-Gly.

and Nle-UPOC were toxic to this yeast. (iv) When chitin synthetase was assayed in permeabilized whole cells, Leu-polyoxin D strongly inhibited the enzyme (unpublished data). This is in contrast to findings in a mixed membrane fraction (Fig. 3). Mixed membrane fractions did not contain peptidase activity, whereas permeabilized cells were equivalent in enzyme content to a cell extract. Thus, it is reasonable to conclude that Leu-polyoxin D and Leu-Nle-UPOC behave as prodrugs which deliver toxic species inside the cell.

The fact that Leu-Nle-UPOC effectively inhibits Met_3 uptake into *C. albicans* H-317 (Fig. 6) suggests that it enters the cell by the peptide transport system. The case of Leu-polyoxin D is more difficult to evaluate. Clearly, it has a poor affinity for the peptide transport system, as judged by its inability to prevent Met_3 transport. Yet its biological effects are reversed by the presence of tryptone in the growth medium. Further experiments are necessary to resolve these seemingly contradictory observations.

Investigations on cell viability indicate that only polyoxin D, Leu-polyoxin D, and Nle-UPOC kill *C. albicans* and that morphological changes are induced at drug concentrations lower than those that cause cell death. We believe that the differences observed between viability and MEC measurements may reflect the stability of various compounds to intracellular hydrolysis. Peptidases present in the cell can degrade all derivatives except for UPOC and polyoxin D. Thus, Leu-Nle-UPOC may first be hydrolyzed to Leu and the active species Nle-UPOC, which is then in turn hydrolyzed to Nle and the inactive UPOC. In the light of this degradation process, it seems reasonable that the active compound reaches concentrations high enough to affect cell morphology but not high enough to kill the yeast. In contrast, Leu-polyoxin D is cleaved to active polyoxin D, which is not further degraded. Therefore, it can kill *C. albicans*. The differences in the viability measurements between polyoxin D and Leu-polyoxin D could be due to different intracellular concentrations of active moiety achieved by these compounds or by protection resulting from the release of Leu in the cell.

In conclusion, we have successfully prepared prodrugs of polyoxin which can markedly affect growth and morphology of *C. albicans* in culture. One of our prodrugs, Leu-Nle-UPOC, can enter the cell via the peptide transport system. Its failure to kill *C. albicans* can be traced to the intracellular lability of the Nle-UPOC linkage, which prevents the buildup of sufficient intracellular concentrations of the toxic moiety. Despite this problem, our findings suggest that a polyoxin with a stable X-UPOC linkage could enter the cell through a peptide transport system and be converted to an active drug. It is clear that chitin synthetase inhibitors have potential as anticandidal agents.

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LITERATURE CITED

1. **Becker, J. M., N. L. Covert, P. Shebagamurthi, A. S. Steinfeld, and F. Naider.** 1983. Polyoxin D inhibits growth of zoopathogenic fungi. Antimicrob. Agents Chemother. **23**:926–929.
2. **Becker, J. M., and F. Naider.** 1980. Transport and utilization of peptides by yeast. p. 257–280. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley & Sons, Inc., New York.
3. **Bowers, B., G. Levin, and E. Cabib.** 1974. Effect of polyoxin D on chitin synthetase and septum formation in *Saccharomyces cerevisiae*. J. Bacteriol. **119**:564–575.
4. **Chiew, Y., M. Shepherd, and P. Sullivan.** 1980. Regulation of chitin synthesis during germ-tube formation in *Candida albicans*. Arch. Microbiol. **125**:97–104.
5. **Damadoran, N. P., G. H. Jones, and J. G. Moffatt.** 1971. Synthesis of the basic nucleotide skeleton of the polyoxin complex. J. Am. Chem. Soc. **93**:3812–3814.
6. **Endo, A., K. Kakiki, and T. Misato.** 1970. Mechanism of action of the antifungal agent polyoxin D. J. Bacteriol. **104**:189–196.
7. **Hart, P. D., E. Russell, and J. S. Remington.** 1969. The compromised host and infection. II. The deep fungal infections. J. Infect. Dis. **120**:169–191.
8. **Hori, M., K. Kakiki, S. Suzuki, and T. Misato.** 1971.

Studies on the mode of action of polyoxins. III. Relation of polyoxin structure to chitin synthetase inhibition. Agric. Biol. Chem. **35**:1280–1291.

9. **Isono, K., T. Azuma, and S. Suzuki.** 1971. Polyoxin analogs. I. Synthesis of aminoacyl derivatives of 5′-amino-5′-deoxyuridine. Chem. Pharm. Bull. **19**:505–512.
10. **Logan, D. A., J. M. Becker, and F. Naider.** 1979. Peptide transport in *Candida albicans*. J. Gen. Microbiol. **114**:179–186.
11. **Mitani, M., and Y. Inoue.** 1968. Antagonists of antifungal substance polyoxin. J. Antibiot. **21**:492–496.
12. **Payne, J. W.** 1980. Transport and utilization of peptides by bacteria, p. 211–256. *In* J. W. Payne (ed.) Microorganisms and nitrogen sources. John Wiley & Sons, Inc., New York.
13. **Ringrose, P. S.** 1980. Peptides as antimicrobial agents, p. 641–692. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources, John Wiley & Sons, Inc., New York.
14. **Wolfinbarger, L., Jr.** 1980. Transport and utilization of peptides by fungi, p. 281–300. *In* J. W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley & Sons, Inc., New York.