[9: R_f 0.07; (S)-proxyphylline (1a): R_f 0.55]. The solvent was removed in vacuo, and the residue was transferred to a separatory funnel with CH_2Cl_2 (50 mL), which was washed with 0.5 M HCl (4 mL) to remove pyridine and colored, water-soluble material. The aqueous phase was extracted twice with CH_2Cl_2 (75 mL), and the combined extracts were washed with 1 M KOH (3 mL) to remove remaining theophylline (9). The extract was dried over anhydrous Na_2SO_4 and evaporated in vacuo to afford a solid residue (774 mg), which was recrystallized from absolute ethanol (2 mL): yield 588 mg (70%); mp 139–149 °C. A second recrystallization from absolute ethanol (3 mL) yielded 162 mg: $[\alpha]^{20}_{589}$ +67.3°, $[\alpha]^{20}_{578}$ +70.3°, $[\alpha]^{20}_{546}$ +80.0°, $[\alpha]^{20}_{436}$ +137°, $[\alpha]^{20}_{365}$ +250° (c 3.0, $CHCl_3$). The crystalline material and the combined, concentrated, mother liquors (576 mg) were separately esterified with (−)-camphanoyl chloride (3; 195 and 737 mg, respectively) in pyridine (2.4 and 8.8 mL, respectively) as previously described, affording 265 (93) and 972 mg (96%) crude product, respectively.

A portion (20.8 mg) of the camphanates present in the former lot was purified by preparative TLC, and the esters were eluted together (18.9 mg). Only traces of the more polar ester corresponding to that from (R)-proxyphylline (1b) could be detected on the TLC plate. The ^1H NMR spectrum (400 MHz) of the purified diastereoisomeric esters allowed an assessment of the ratio of the two esters. A comparison of the integral of the CH_3 singlet at δ 0.92 with that of a small CH_3 singlet at δ 0.78 indicated a ratio 2a to 2b of 98.6:1.4. The remainder of the former lot of camphanates and the latter (972 mg) were recrystallized separately from MeOH (15 and 60 mL, respectively), furnishing chromatographically pure (S)-proxyphylline camphanate (2a): yield 906 mg; mp 203–204.5 °C; for $[\alpha]^{20}$, see Table II; for MS, ^1H NMR, and R_f, see above under (S)-proxyphylline camphanate (2a). Anal. ($C_{20}H_{26}N_4O_6$) C, H, N. The camphanate 2a (873 mg, 2.09 mmol) was hydrolyzed [129.3 mg of KOH (2.31 mmol), 67 mL of MeOH; 12 mL of H_2O] and worked up as previously described, except for one additional washing of the $CHCl_3$–extract (270 mL) with 3% $NaHCO_3$ (10 mL). Crystallization from ethyl acetate furnished (S)-proxyphylline (1a) (416 mg, 84%): mp 150–151.5 °C; for $[\alpha]^{20}$, see Table II; for MS, ^1H NMR, and R_f, see above under (S)-proxyphylline.

Acknowledgment. The authors are indebted to the Norwegian Research Council for Science and the Humanities for providing mass spectroscopy and NMR facilities. The technical assistance of Kari Frogner is gratefully acknowledged.

Registry No. (±)-1, 86480-51-5; 1a, 86540-95-6; 1b, 86540-96-7; 2a, 86480-52-6; 2b, 86540-97-8; 4, 687-47-8; 5, 4254-15-3; 6, 16088-60-1; 7a, 16088-61-2; 7b, 60434-72-2; 8a, 16088-62-3; 8b, 15448-47-2; 9, 58-55-9; cyclic 3′,5′-nucleotide phosphodiesterase, 9040-59-9.

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Design of Anticandidal Agents: Synthesis and Biological Properties of Analogues of Polyoxin L†

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Six analogues of polyoxin L were synthesized from uridine. All of these analogues inhibited chitin synthetase from *Candida albicans*. Derivatization of the amine terminus of the polyoxin analogues resulted in loss of activity, and analogues containing aromatic amino acid residues were the most efficient inhibitors of chitin synthetase. The concentration of tryptophanyl uracil polyoxin C, 8, which caused 50% inhibition of chitin synthetase activity, was 1.6×10^{-6} M. This was virtually identical with the activity found for polyoxin D. None of the inhibitors effectively competed with the entry of $(Met)_3$ into *C. albicans*. All of the analogues caused severe morphological distortions of the yeast in culture, and a number of analogues killed *C. albicans* at millimolar concentrations. The results suggest that chitin synthetase inhibitors may have potential as anticandidal drugs.

The polyoxins are a series of peptidyl nucleoside antibiotics that strongly inhibit chitin synthetase from a spectrum of fungi[1,2] and arthropods.[3,4] They are known to be highly toxic against both phytopathogenic fungi[5] and insects[4] but are not toxic to vertebrates.[6] The absence of detectable toxicity in mammalian systems makes the polyoxins attractive as potential agents against systemic fungal infections. However, previous workers concluded that polyoxins are not active against medically important fungi, such as *Candida albicans*.[6,7] Two reports[8,9] suggested that this lack of activity might result from the inability of the antibiotic to penetrate the cell to the site of chitin synthetase. Support for this suggestion is found in the observations that the polyoxins exhibit similar activities against chitin synthetase preparations from a variety of yeasts, including *Saccharomyces cerevisiae*, but only affect *S. cerevisiae* in culture when present at high concentration.[10] Furthermore, when used in combination

† Abbreviations used in this paper are as follows: Z, benzyloxycarbonyl; DMF, *N,N*-dimethylformamide; ONp, *p*-nitrophenyl ester; NMM, *N*-methylmorpholine; TLC, thin-layer chromatography; *n*-BuOH, *n*-butyl alcohol; HOAc, acetic acid; TFA, trifluoroacetic acid.

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Table I. Synthetic Analogues of Polyoxin L

no.	$[\alpha]^{25}_D$, deg	R_f[b]	yield,[c] %	formula[e,f]	anal.[e,f]
3[a]	+13.94 (*c* 0.33, H_2O)	0.20			
4	+51.27 (*c* 0.16, H_2O)	0.42	50	$C_{20}H_{24}N_4O_8 \cdot 2H_2O$	C, H, N
5	+30.43 (*c* 0.14, H_2O)	0.36	38	$C_{16}H_{24}N_4O_8 \cdot 1.4H_2O \cdot 0.6CF_3COOH$	C, H, N, F
6	+26.61 (*c* 0.11, H_2O)	0.15	53	$C_{16}H_{24}N_6O_9 \cdot 2H_2O \cdot CF_3COOH$	C, H, N, F
7	+37.89 (*c* 0.19, H_2O)	0.31	45	$C_{19}H_{22}N_4O_9 \cdot 0.6H_2O \cdot HCOOH$	C, H, N
8	+37.78 (*c* 0.18, H_2O)	0.36	40	$C_{21}H_{23}N_5O_8 \cdot 2H_2O \cdot 0.8CF_3COOH$	C, H, N, F
9	+14.12 (*c* 0.17, H_2O)	0.38	22[d]	$C_{25}H_{26}N_4O_{10} \cdot 2H_2O \cdot CF_3COOH$	C, H, N

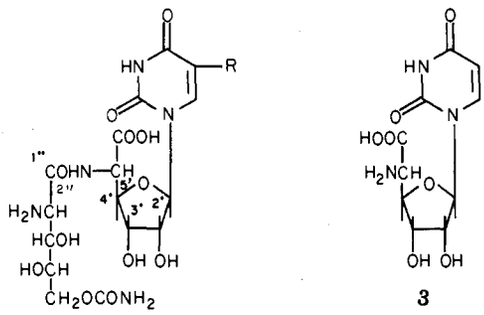
[a] Compound 3 is the starting material for 4–9 and was prepared as the formate salt. Damodaran et al. report $[\alpha]^{23}_D$ +17.6° (*c* 0.49, H_2O) for the free base.[15] [b] Measured on silica thin layers with 1-butanol/acetic acid/water (4:1:2) as the mobile phase. [c] Represents the overall yield of coupling and hydrogenolysis to a product that was greater than 98% homogeneous on HPLC with the exception of 9. [d] The intermediate Z-protected derivative could not be isolated as a solid. Compound 9 was purified directly by semipreparative HPLC. [e] With the exception of 7, all analyses agree with calculated values to ±0.4%. Anal. Calcd for 7: C, 47.35; H, 5.01; N, 11.04. Found: C, 47.61; H, 5.51; N, 10.75. [f] Compounds 5, 6, 8, and 9 were purified on HPLC with a $CH_3OH/H_2O/CF_3COOH$ mobile phase.

with membrane-permeabilizing drugs, polyoxin D did affect *S. cerevisiae* at lower doses.[9] These results and the apparent large and diverse group of chitin-containing organisms that are susceptible to polyoxin inhibition suggested to us that the polyoxins might in fact be active against chitin synthetase from *C. albicans* but cannot reach the enzyme that is located within the cell membrane. Preliminary studies from our laboratory showed that polyoxin D at a concentration of 1×10^{-5} M inhibited the incorporation of labeled UDP-[^{14}C]GlcNAc into chitin from *C. albicans* H317.[11] This level of activity for polyoxin D against chitin synthetase from *C. albicans* was similar to that found for various polyoxins against the enzyme from *S. cerevisiae*[12] and phytofungi.[1,8,13] Unexpectedly, we also found that high concentrations (>1 mM) of polyoxin D caused extensive morphological changes and cell death of *C. albicans*.[11]

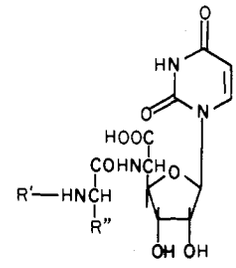
The goal of our research is to structurally alter the polyoxin molecule so as to allow it to efficiently penetrate *C. albicans* and inhibit chitin synthetase. In this paper we report the total synthesis of a series of polyoxin analogues with modified peptidyl moieties and the corresponding structure–activity relationships.

Chemistry. The vast majority of biological investigations on polyoxins have utilized polyoxin D (1). Polyoxin L (2) differs from polyoxin D, Chart I, only in that the 5-carboxyl is replaced with a hydrogen atom.[14] The compounds that we synthesized, **4–9**, were prepared from uridine and are, thus, analogues of polyoxin L. Our first objective was to prepare 1-(5′-amino-5′-deoxy-β-D-allofuranosyluronoyl)uracil, **3**, which we called uracil polyoxin C. This intermediate corresponds to the product that would be obtained by alkaline hydrolysis of the dipeptidyl bond of polyoxin L. Starting with 2′,3′-*O*-cyclohexylideneuridine, **3** was synthesized by the procedure of Damodaran et al.[15] This intermediate was then used to prepare a variety of analogues by selectively reacting the 5′-amino group of **3** with *p*-nitrophenyl esters of benzyloxycarbonyl (Z) protected amino acids in DMF–water at room temperature.[16] The yield of the Z-protected polyoxin

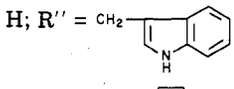
Chart I. Structural Formulas of Polyoxins



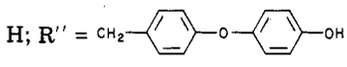
1, R = COOH
2, R = H



4, R′ = H; R″ = CH_2CH_2-C_6H_5
5, R′ = H; R″ = $CH_2(CH_2)_2CH_3$
6, R′ = H; R″ = $CH_2(CH_2)_2NHCONH_2$
7, R′ = H; R″ = CH_2-C_6H_4-OH



8, R′ = H; R″ = CH_2



9, R′ = H; R″ = CH_2

analogues isolated ranged from 50 to 58%. They were deblocked in yields ranging from 75 to 90% by transfer hydrogenation with palladium black–formic acid in methanol.[17] Most analogues were synthesized starting with 100 mg of **3**, and we were able to recover 40 to 50 mg of product after this two-step synthesis. Many of the polyoxin analogues prepared by these methods were greater than 98% pure as judged by HPLC. Compounds **5**, **6**, **8**, and **9** were further purified by using semipreparative reversed-phase HPLC with $CH_3OH/H_2O/CF_3COOH$ mixtures as eluent, to a final purity of greater than 99.8%. The physical properties of the polyoxin analogues are given in Table I. All compounds were

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Table II. Biological Effects of Polyoxins

no.	biological effects: ID_{50},[a] M	MEC,[b] mM	viability,[c] mM
1	1.8×10^{-6}	0.02	1
3	$>10^{-4}$	8.00	>8
4	3.8×10^{-6}	0.06	>8
5	1.4×10^{-5}	0.17	4
6	6.8×10^{-5}	0.50	>8
7	4.9×10^{-6}	8.00	>8
8	1.7×10^{-6}	0.13	>8
9	1.6×10^{-5}	2.00	4

[a] The molar concentration of compound that inhibited by 50% the activity of chitin synthetase as measured by the incorporation of *N*-acetylglucosamine into chitin. [b] The minimum effective concentration (MEC) is the lowest concentration at which some (5%) morphologically abnormal cells are observed under microscopic observation. [c] The concentration at which 50% of the cells incubated with the compound were not able to form colonies when replated.

characterized by either 80- or 300-MHz proton NMR. They exhibit chemical-shift values in conformity with those reported in the literature and expected for the peptidyl and nucleoside portions of the molecule.

Biological Results and Discussion

Effect of Polyoxin Analogues on Chitin Synthetase from *Candida albicans* H-317. Activity of chitin synthetase was assayed by using a mixed membrane fraction[18] from *C. albicans* H-317, a clinical isolate obtained from The Center for Disease Control, Atlanta, GA. Trypsin was incubated with the chitin synthetase to activate the enzyme, and activity was measured by following the incorporation of radioactivity from UDP-[^{14}C]GlcNAc into chitin. In a preliminary study, we reported that 1 inhibited chitin synthetase of strain H-317.[11] Using similar procedures we determined dose–response curves for each synthetic analogue of polyoxin, and ID_{50} values were derived over the concentration range of 10^{-4} to 10^{-7} M (Table II). A typical dose–response curve is presented for compound **4** (Figure 1). The only compound without a peptidyl moiety, **3**, was found to be inactive ($ID_{50} > 10^{-4}$ M), substantiating the need of a peptide group for significant activity. Nevertheless, substantial structural variations in the peptide chain can be tolerated without sacrificing activity, as evidenced by the wide variety of residues represented in compounds **4–9**. The most active analogues, **4**, **7**, and **8**, were approximately equal in activity to polyoxin D. The amino acid residues in these molecules are all very hydrophobic, suggesting that the lipophilicity potentiates the activity of these polyoxins. Although large structural changes are tolerated in the amine terminal residue, derivitization of the C_2''-amino group resulted in inactive compounds. For example, the precursors of compounds **5**, **6**, and **8**, which contain a benzyloxycarbonyl protecting group on the C_2''-amine, did not inhibit chitin synthetase at concentrations exceeding 10^{-4} M. Compound **6**, which has a long alkyl chain ending with a ureido group, mimics the carbamoylpolyoxamic acid side chain of the natural polyoxins.[14] Although active ($ID_{50} = 6.8 \times 10^{-5}$ M), this analogue was not as effective against chitin synthetase of *C. albicans* as many other derivatives. Findings similar to the above were reported for the activity of several polyoxin analogues against chitin synthetase from phytofungi.[16,19-21]

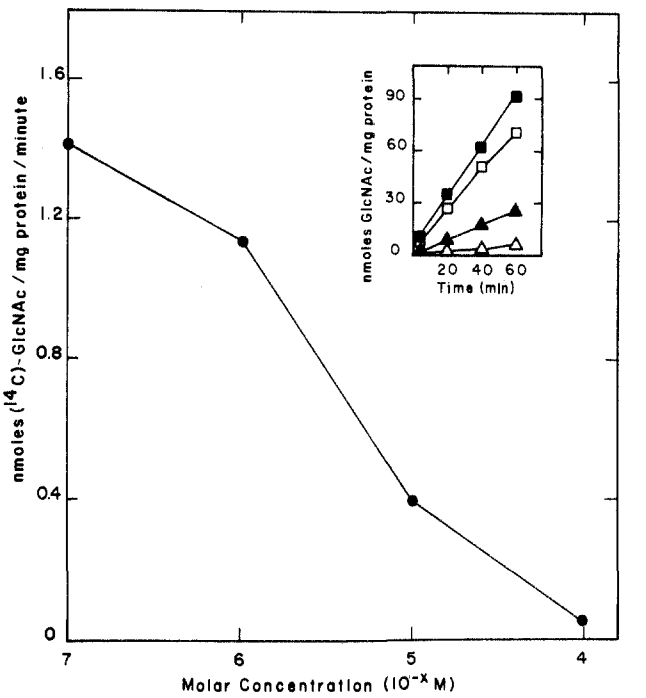

Figure 1. Dose–response curve for homophenylalanyl uracil polyoxin C (**4**) as measured by the chitin synthetase assay. The dose–response curve (●) presents the initial velocity of each time course assay at the respective molar concentration of the inhibitor. Half-maximal activity of chitin synthetase in the presence of **4** corresponds to 0.73 mol of GlcNAc mg^{-1} min^{-1} and occurs at a molar concentration of 3.8×10^{-6}, which we define as the ID_{50}. Insert shows the time course of [^{14}C]GlnNAc incorporation into chitin in the presence of 10^{-4} (△), 10^{-5} (▲), 10^{-6} (□), and 10^{-7} M (■).

Inhibition of *C. albicans* H-317 by Polyoxins. We have found that when incubated with *C. albicans*, polyoxin D inhibits growth and causes morphological changes.[11] Cells that do grow form extensive chains with bulbous cells at the end of the chain that are susceptible to osmotic changes in the incubation medium. The primary septum requires chitin as a component, and we attribute the morphological aberrations to inhibition of septum formation between mother and daughter cell. The effects are observed both in the yeast and mold phases. Similar effects are observed with many of the polyoxin analogues. In particular, extensive morphological changes result from the incubation of either **5** or **8** with *C. albicans*. All of the drugs were evaluated in order to determine the minimum effective concentration (MEC), the lowest concentration at which some (5%) morphologically abnormal cells are observed under microscopic observation (Table II). The effect of the drugs on cell viability was established by determining the number of colonies formed after incubation of a known number of cells with various amounts of the synthetic polyoxins. For example, the results of MEC and viability tests on **5** were 0.17 and 5.00 mM, respectively, compared to 0.02 and 1.00 mM for **1** (Table II). If one plots the log ID_{50} values vs. log MEC for the compounds in Table II, there is a reasonable correlation, with the exception of compound **7**. However, there is no correlation between chitin synthetase inhibition results and viability studies. Whereas many analogues had ID_{50} values

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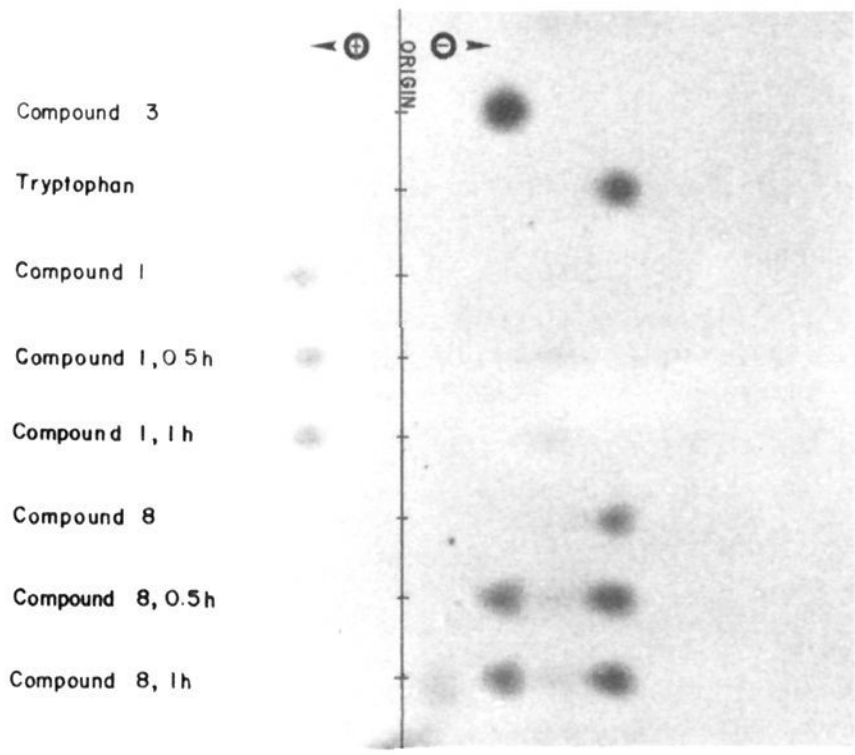

Figure 2. Paper electrophoresis at pH 3.5 of polyoxin D (1) and tryptophanyl uracil polyoxin C (8) after incubation with cell extract for 30 and 60 min. Markers for cell extract, uracil polyoxin C (3), and tryptophan are represented adjacent to markers for 1 and 8.

similar to polyoxin D, only **5** and **9** show 50% killing of cells at a concentration similar to **1**.

Inhibition of [^{14}C]Trimethionine Transport by Polyoxin Analogues. Data in the literature have indicated that peptones present in the culture medium prevent the toxic effects of polyoxin D (**1**) on fungi.[9,22] In a recent investigation we found that 1% tryptone and, to a lesser degree, yeast extract (1%) and peptones (1%) prevented the toxic effects of **1** on *Candida albicans*.[11] These results suggest that peptides and the polyoxins may share the same transport system. To test this possibility, we examine the ability of compounds **1** and **3–9** to compete with the uptake of [^{14}C]trimethionine into *C. albicans*. This tripeptide was previously found to be a substrate for the peptide transport system in *C. albicans*.[23] Competition studies were conducted by measuring the initial rate of uptake of radiolabeled trimethionine into *C. albicans* H-317 in the presence of 5-, 10-, and, in a few cases, 50-fold excess of **1** and **3–9**. Under the conditions of assay using 10-fold excess of the polyoxins, we observed a lowering of the initial uptake rate of trimethionine by a maximum of 22% (compound **7**). In most cases, competition was between 0 and 10%, which falls within the experimental limits of the transport assay. These results suggest that compounds **1** and **3–9** have a very low affinity for the peptide transport system in *C. albicans* H-317. They do not, however, exclude the possibility that the polyoxins use this transport system for entry into the cell.

Metabolism of Polyoxin D and Polyoxin Analogues by *C. albicans* H-317. Cells of *C. albicans* H-317 were grown in culture, harvested by centrifugation, and broken by grinding in a blender with glass beads. Portions of the cell extracts obtained by this procedure were then incubated with **1** and the polyoxin analogues **4–9** at 37 °C for time periods of 0, 30, and 60 min. The compounds were spotted on Whatman paper and run on high-voltage electrophoresis along with standards (Figure 2). Interestingly, most dipeptidyl analogues are rapidly metabolized to the inactive **3** and the corresponding amino acid by cell extracts of *C. albicans* H-317 (Figure 2). In contrast, under identical conditions polyoxin D is resistant to further degradation (Figure 2). Control studies showed that in the absence of cell extract all compounds were stable under the incubation conditions. If the hydrolysis studies reflect physiological events in the intact cell, they may account for the apparent discrepancies between effects of analogues on cell viability and chitin synthetase. Thus, the synthetic analogues have little effect on cell viability because they are cleaved to inactive products by intracellular enzymes. This hypothesis may also explain why several analogues effect cell morphology (MEC) but not viability. It is possible that morphological aberrations reflect the onset of drug activity, while cell death requires longer incubations with the intact drug.

Conclusion

We have found that polyoxin D and a series of synthetic analogues, **4–9**, are active against chitin synthetase from *C. albicans* and are also toxic in relatively high doses against whole cells grown in culture. Our results show that the peptidyl portion of the polyoxin molecule can be significantly altered without losing activity against chitin synthetase. Thus, we believe that there is an excellent possibility of designing active molecules that can penetrate the cell more readily. Finally, the present report suggests that the development of a polyoxin analogue that is efficiently transported into *C. albicans* and is stable to metabolic degradation inside this yeast should provide a superior anticandidal agent.

Experimental Section

All amino acids were of the L configuration. Polyoxin D was isolated as the trifluoroacetyl salt as described previously.[24] Melting points were determined by capillary method and are uncorrected. All solvents used were of analytical grade supplied by Fisher Scientific and Eastman. Thin-layer chromatography of all the intermediates and polyoxins were carried out on silica gel plates (Brinkmann), and the spots were detected by UV light and/or ninhydrin staining. High-performance liquid chromatography (analytical) was carried out on a Waters system consisting of a M-6000 solvent-delivery unit and a U6K injector, coupled to a Waters 450 variable-wavelength UV monitor with an 8-μL flow-through cell. The μBondapak C_{18} column (both analytical and semipreparative) was also from Waters Associates. Solvents used were of HPLC grade, and water was glass distilled.

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. All the polyoxins have various amounts of water and CF_3COOH (or HCOOH) associated with them (Table I). 300 MHz, NMR, spectra were recorded on a Nicolet instrument at the Rockefeller University, New York.

Synthesis. The conjugation of various amino acids with uracil polyoxin C (**3**) was carried out by the active ester coupling method of peptide synthesis. A detailed procedure is given below for the synthesis of **4**. Other polyoxins were synthesized by a similar method using appropriate quantities of the reagents. The yields obtained and some physical constants are summarized in Table I.

Homophenylalanyl Uracil Polyoxin C Formate Dihydrate (4). To a solution of uracil polyoxin C formate[15] (100 mg, 0.3 mmol) and benzyloxycarbonyl-L-homophenylalanine *p*-nitrophenyl ester (143 mg, 0.33 mmol) in dimethylformamide (1.5 mL) containing water (0.3 mL) was added *N*-methylmorpholine (0.066 mL, 0.6 mmol), and the mixture was stirred at room temperature for 24 h. The progress of the reaction was monitored by TLC (*n*-BuOH/HOAc/H_2O, 4:1:2). When most of the nucleophile had reacted (85%), the reaction mixture was acidified with acetic acid,

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the solvents were evaporated in vacuo, and the crude product was precipitated by the addition of ether. The solid was filtered, washed with ether, and dried. It was again dissolved in DMF and precipitated with 2% aqueous acetic acid to remove any unreacted **3** and salts. The yield of product was 92 mg (53%). It was used without further purification to prepare **4**. To a solution of benzyloxycarbonyl-L-homophenylalanyl uracil polyoxin C (70 mg) in CH_3OH (3 mL) was added Pd black (30 mg). Formic acid (90%, 0.3 mL) was then added with stirring at room temperature. After 30 min, when TLC indicated complete hydrogenolysis, the catalyst was removed by filtration through Celite, and the filtrate was evaporated to dryness. The residue was dissolved in water (10 mL) and filtered through a microfilter (0.45 μm), and the filtrate was freeze-dried: yield 56 mg (95%); the $[\alpha]^{25}_D$ and R_f values are reported in Table I; NMR (Me_2SO-d_6) δ 5.59 (d, J = 7.9 Hz, 1, C_5 H), 5.79 (d, J = 6.2 Hz, 1, $C_{1'}$ H), 7.22 (m, 5, Ar H), 7.81 (d, J = 7.9 Hz, 1, C_6 H), 8.19 (d, J = 8.2 Hz, 1, amide NH). For C, H, and N analysis, the freeze-dried sample was further dried by heating in vacuum at a temperature of 60 °C for 24 h. Under these conditions, the molecule loses all the HCOOH but still retains two water molecules bonded to it. Anal. ($C_{20}H_{24}O_8N_4 \cdot 2H_2O$) C, H, N.

Biochemical Methods. Chitin Synthetase Assay. Chitin synthetase activity obtained in a mixed membrane fraction (mmf)[18] from *C. albicans* H-317 was assayed in the presence and absence of polyoxin compounds by measuring the incorporation of *N*-acetylglucosamine (GlcNAc) into chitin. For assay of total chitin synthetase activity, the enzyme was activated with trypsin in a reaction assay containing 125 μL of mmf (5.17 mg of protein/mL); 25 μL of 4 mM ATP; 15 μL of 0.5 M imidazole buffer, pH 6.5; 5 μL of trypsin (2 mg/mL); and 35 μL of distilled H_2O. After incubation at 37 °C for 10 min, 5 μL of trypsin soybean inhibitor (3 mg/mL), 10 μL of 0.8 M GlcNAc, 5 μL of 50 mM UDP-[^{14}C]GlcNAc (200000 cpm/mol), and 25 μL of H_2O for the control, or polyoxin analogues at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M was added for each assay. Aliquots 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 precipitates 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 counted by liquid scintillation for the incorporation of radioactivity into chitin.

Uptake of Radioactive Trimethionine into *C. albicans* H-317. The uptake of L-methionyl-L-methionyl-L-[^{14}C]methionine was determined by the procedure of Logan et al.[23] For competition studies, compounds **1** and **3**–**9** were dissolved in distilled water and added simultaneously with radioactive trimethionine. Initial rates of transport were determined as nanomoles of peptide taken up per milligram dry weight of cells.

Preparation of Cell Extract. Cells of *Candida albicans* H-317 were harvested at late logarithmic phase of growth and washed twice with distilled water. For each gram of yeast cells (wet weight) was added 1.5 to 2.0 mL of 0.1 M NaCl; 0.1 M Tris-HCl, pH 7.2; and 5.0 g of glass beads (Minnesota Mining & Manufacturing Co., 50–70-μm diameter) 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 (25400*g* for 30 min). The crude extract was then dialyzed against 100 vol of 0.1 M NaCl and 0.1 M Tris-HCl, pH 7.2, for 24 h, with a buffer change after 3 h. After 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/mL) from *C. albican* 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 H_2O). At intervals of 30 and 60 min, 10 μL was removed from the reaction mixture and applied to 3MM Whatman paper for subsequent electrophoresis.

Electrophoresis. Electrophoresis was carried out in a Model LT-36 electrophoresis tank, E.C. 123 coolant, and an HV-5000 power supply (Savant Instruments, Hicksville, NY). 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 vol/cm for 2 h. After electrophoresis, the paper was dried, dipped in a solution of ninhydrin (0.5%, w/v) in 95% aqueous acetone, and developed by heating in a ventilated oven.

Determination of Minimum Effective Concentration (MEC) and Viability. *C. albicans* H-317 was grown at 37 °C with stirring in yeast nitrogen base (Difco Co., Detroit, MI) containing 0.5% isoleucine and 5.0% glucose. Cells were harvested by centrifugation at early logarithmic phase of growth (approximately 60 Klett units using blue filter, Klett Summerson photoelectric colorimeter). The cells were washed with sterile yeast nitrogen base and resuspended to 3×10^6 cells/mL for use as the inoculum for microtiter plates (Corning Glass Works, Corning NY). Yeast nitrogen base, 50 μL, was added to microtiter wells, various polyoxin analogues or polyoxin D in yeast nitrogen base, 50 μL, was added to the first well, and the concentration of drug was adjusted by twofold serial dilutions. To each well was then added 25 μL of inoculum. By this procedure each well contained various concentrations of the drug and 1×10^6 cells/mL in a total volume of 75 μL. The microtiter plates were incubated at 37 °C in a humid chamber with slight agitation. After 48 h of incubation, portions were removed from each well and examined microscopically. The MEC is defined as the lowest concentration of drug that resulted in some (~5%) morphologically abnormal cells at 48 h. In addition, the number of potential viable units was determined by hemocytometer counting, and a portion was removed from each well and serially diluted through yeast nitrogen base. Portions from each of these dilutions were spread on potato dextrose agar (Difco Corp., Detroit, MI) and incubated for 2 days at 37 °C. The number of viable colonies was determined by direct counting. Percent viability was calculated by comparing the number of viable colonies to the number of potential viable cells by direct counting. Under our conditions, cells not treated with an antibiotic gave nearly 100% of the expected colonies on plating.

Acknowledgment. This work was supported by a grant from the National Institute of Allergy and Infectious Disease (AI-14387) and the PSC-BHE Awards Program of CUNY. The 300-MHz NMR spectral studies at Rockefeller University were supported in part by NSF Grant PCM-7912083 and the Camille and Henry Dreyfus Foundation.

Registry No. 1, 22976-86-9; **2**, 22976-90-5; **3**, 24695-48-5; **4**, 86632-63-5; **5**, 86632-64-6; **6**, 86632-65-7; **7**, 86632-66-8; **8**, 86632-67-9; **9**, 86632-68-0; trimethionine, 14486-15-8; chitin synthetase, 9030-18-6.