addition of the cuprate derived from (*E*)-1-(tributylstannyl)-4-methyl-4-[(trimethylsilyl)oxy]-1-octene[19] (7.2 mmol) to cyclopentenone **45** (3.5 mmol) by the procedure described above for **53** furnished an amber oil. The trimethylsilyl groups of the conjugate adduct were removed (HOAc–THF–H_2O, 4:2:1) and the resulting brown oil was applied to a silica gel dry column (3 × 55 in.; 2:3 EtOAc–benzene + 2% acetic acid; 700 mL of eluant was collected). From the column at R_f 0.35–0.45 was isolated 350 mg (23%) of **52** as a yellow oil: NMR δ 5.80–5.23 (m, 4 H, C-13 and C-14 H, C-5 and C-6 H), 3.73 (m, 2 H, $HOCH_2$), 3.70 (s, 3 H, $-COOCH_3$), 3.16–2.63 (m, 3 H, $-CH_2S-$, C-11β H), 1.23 (s, 3 H, $-CH_3$), 0.93 (t, 3 H, C-20 H). MS Calcd for $C_{22}H_{34}O_4$ (M – $HOCH_2CH_2SH$): 362.2457. Found: 362.2450.

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Anticandidal Activity of Pyrimidine–Peptide Conjugates

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The ability of conjugates of peptides and 5-fluorocytosine or 5-fluoroorotic acid to enter *Candida albicans* was investigated. A number of conjugates of 5-fluoroorotic acid and peptides were synthesized using 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline as the coupling agent. Orotyl-L-leucyl-L-leucine, 5-fluoro-4-(*N*-succinamoyl-L-alanyl-L-leucine)-2(1*H*)-pyrimidinone [a 5-fluorocytosine derivative], and 5-fluoroorotyl-L-leucyl-L-leucine all inhibited the uptake of trimethionine into *C. albicans* WD 18-4. Inhibition by 5-fluoroorotyl-L-leucyl-L-leucine was competitive as judged using double-reciprocal plots. Evaluation of minimun inhibitory concentrations of peptide–5-fluorocytosine conjugates suggest that these conjugates enter *C. albicans* in the intact form. These results provide the first experimental evidence that peptides can carry pyrimidines into a eukaryote.

Many potential therapeutic agents are ineffective because of the failure of the active molecules to permeate the cell membrane. Attempts to overcome this problem include modification with lipophilic groups, attachment to macromolecules, and entrapment in liposomes. Another approach employs the use of active-transport systems to bring potentially cytotoxic drugs into cells. The advantage of this approach is that it can result in a 100- to 1000-fold concentration of the toxic agent inside the cell.

Several investigators have demonstrated that peptides can carry normally impermeant molecules into microorganisms. Fickel and Gilvarg[1] with *Escherichia coli* and Ames and co-workers[2] using *Salmonella typhimurium* showed that the metabolic intermediates homoserine phosphate and histidinol phosphate, respectively, could enter bacteria when incorporated into a peptide. Toxic amino acid analogues such as ethionine were smuggled into *S. typhimurium* as peptide residues,[2] and recently L-1-aminoethylphosphonic acid was incorporated into a peptide and brought into both Gram-negative and Gram-positive bacteria through the dipeptide transport system.[3] Although the amino acid mimetic L-1-aminoethylphosphonic acid itself was inactive, it was responsible for broad antibacterial activity when introduced as a dipeptide. Peptides have also been shown to carry a number of other unusual amino acids across the cell membrane of *C. albicans*.[4]

Previous studies from our laboratory have attempted to use peptides in the design of drugs for *Candida albicans*.[5-8] We have described the structural specificity of the peptide transport system in one strain of *Candida*[5,8] and have developed synthetic procedures to conjugate peptides to 5-fluorocytosine (5-FC).[7] Several peptide–5-fluorocytosine conjugates strongly inhibited the growth of a number of yeast strains. At the time of that report, the poor stability of these conjugates in solution prevented us from demonstrating whether they entered *C. albicans* via the peptide transport system. In this article, we extend our studies on peptide–5-fluorocytosine conjugates and report on peptide conjugates with 5-fluoroorotic acid. Using both microbiological assays and competition with the uptake of radioactive trimethionine, we provide evidence that conjugates of peptides and pyrimidines can enter yeast via the peptide transport system.

Results

Synthesis of Peptide–5-Fluoroorotic Acid Conjugates. Previous attempts to prepare ester or amide derivatives of the 6-carboxyl group of orotic or fluoroorotic acid have used the corresponding acid chloride as the reactive intermediate. Many of these syntheses were conducted in nonpolar solvents such as $CHCl_3$, and in a typical procedure the orotic acid or its acid chloride and alcohol

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Table I. Summary of Properties of Orotyl and 5-Fluoroorotyl Derivatives

compd	synth proced	yield, %	mp, °C	$[\alpha]^{25}_D$, deg	R_f (solv syst)[a]	anal.
			(A) Esters			
Ort-Gly-OMe	method B	82	232 (dec)		0.3 (A) 0.65 (B)	C, H, N
Ort-Gly-O-*t*-Bu	method B	87	189 (dec)		0.3 (A) 0.67 (B)	C, H, N
Ort-Gly-Gly-OBzl	method B	83	240 (dec)		0.6 (C)	C, H, N
Ort-Gly-Gly-O-*t*-Bu	method B	71	191-193 (dec)		0.67 (C)	C, H, N
Ort-Leu-Leu-OMe	method A	25	218	−32.3 (*c* 0.38, EtOH)	0.3 (A) 0.71 (B)	C, H, N
	method B	85	218		0.3 (A) 0.71 (B)	C, H, N
Ort-Met-Met-OMe	method A	24	132	+34.5 (*c* 0.15, EtOH)	0.17 (A) 0.68 (B)	C, H, N, S
5-FOrt-Leu-O-*t*-Bu	method B	79	160	−38.6 (*c* 0.2, EtOH)	0.44 (D)	C, H, N, F
5-FOrt-Gly-Gly-OBzl	method B	53	238 (dec)		0.6 (C) 0.77 (E)	C, H, N, F
5-FOrt-Leu-Leu-O-*t*-Bu	method B	98[b]	152	−25.4 (*c* 0.5, EtOH)	0.35 (A) 0.76 (B)	C, H, N, F
			(B) Free Acids			
Ort-Gly-Gly-OH	HCl/HOAc	48	240 (dec)		0.7 (F)[c]	C, H, N
Ort-Leu-Leu-OH	method A	79	98 (dec)	−19.1 (*c* 0.11, H_2O)	0.7 (F)	C, H, N
	HCl/HOAc	93				
5-FOrt-Leu-OH	HCl/HOAc	91	225 (dec)	−9.0 (*c* 0.2, H_2O)	0.62 (E)	C, H, N
5-FOrt-Leu-Leu-OH	HCl/HOAc	95	201	−12.0 (*c* 0.11, H_2O)	0.75 (F)[c]	C, H, N, F

[a] On silica thin layers. The solvent systems (in parentheses) were as follows: A = ethyl acetate, B = ethyl acetate/methanol (2:1), C = ethyl acetate/methanol (1:1), D = ether, E = methanol, F = 2-propanol/water (7:3). All residues are of the L configuration. [b] Crude yield purified on high-performance LC. [c] On cellulose thin layers.

are refluxed to drive the reaction.[9] Since such procedures are not particularly suitable for the conjugation of the orotic acids to peptides with retention of chirality of the amino acids, alternative synthetic approaches were developed. We found that conjugation of dipeptides or dipeptide esters with orotic acid could be readily accomplished by reaction of the appropriate nucleophile with orotic acid *N*-hydroxysuccinimide ester (method A).

Surprisingly, when this methodology was extended to 5-fluoroorotic acid, all attempts to prepare the corresponding hydroxysuccinimide ester failed due to steric and/or electronic influences of the fluorine atom in the 5 position of the pyrimidine ring. However, successful conjugation of both orotic acid and 5-fluoroorotic acid with amino acid and dipeptide esters was achieved using 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) as the coupling agent (method B). The reaction was carried out in dimethylformamide at 50–55 °C for 12–24 h. Coupling yields were generally between 80 and 90%. Conjugates containing free carboxyl end groups were prepared from the corresponding *tert*-butyl esters by acidolysis using 1 N hydrogen chloride in acetic acid. Table I summarizes the physical properties of various amino acid and dipeptide conjugates of orotic acid and 5-fluoroorotic acid which we have prepared.

Stability of Peptide–5-Fluoropyrimidine Conjugates. In a previous study we found that peptides conjugated to 5-fluorocytosine via the exocyclic nitrogen were unstable in buffered growth medium (yeast nitrogen base) at pH 7.2.[7] Therefore, prior to evaluating the biological activity of the peptide–orotic acid conjugates we investigated their stability under various conditions. Conjugates of orotic acid with amino acids and dipeptides were completely stable in yeast nitrogen base buffered at pH 7.2. After 2 days in solution at room temperature, neither orotylglycylglycine nor orotylleucylleucine showed any evidence of breakdown as judged using thin-layer chromatography on silica plates with 2-propanol/H_2O (7:3) as the eluent. In addition, boiling of several orotyl–amino acid conjugates in distilled water for 2–3 h did not cause detectable breakdown. Similar results were obtained with conjugates of 5-fluoroorotic acid and amino acids or peptides. These findings show that the amide bond between the 6-carboxyl of orotic or 5-fluoroorotic acid and amino acids or peptides is stable to nonenzymatic hydrolysis.

We also reexamined the stability of conjugates of 5-FC and peptides. We found that in yeast nitrogen base a change in pH from 7.2 to 5.0 caused a marked increase in the stability of several conjugates. For example, 5-fluoro-4-(*N*-succinamoyl-L-alanyl-L-leucine)-2(1*H*)-pyrimidinone [N^4-(Succ-Ala-Leu-OH)-5-FC] has a $t_{1/2}$ = 1.7 h at pH 7.2, $t_{1/2}$ = 16.5 h at pH 6.0, and $t_{1/2}$ = 49.0 h at pH 5.0. Analogous results were obtained with N^4-(Succ-Leu-Leu-OH)-5-FC and with several peptide ester conjugates.

Table II. Competition for Transport of Trimethionine by Various Peptide–Pyrimidine Conjugates[a]

competition	inhibn of trimethionine transport, %
Ort-Leu-Leu	67
Ort-Met-Met-OMe	0
Ort-Gly-Gly	0
N^4-(Succ-L-Ala-L-Leu)-5-FC	55
5-F-Ort-Leu	0
5-F-Ort-Leu-Leu	40

[a] The initial rate of uptake of radioactive trimethionine into *Candida albicans* WD 18-4 was determined as described under Experimental Section. Competitors were added as indicated at 10-fold higher molar concentration to that of trimethionine.

Competition between Pyrimidine–Peptide Conjugates and Met-Met-[^{14}C]Met Transport in *C. albicans*. The ability of pyrimidine–peptide conjugates to utilize the peptide transport system was judged by measuring the initial rate of trimethionine uptake into *C. albicans* WD 18-4 at pH 3.5 in the presence of these derivatives (Table

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Table III. Toxicity of Peptide-5-Fluorocytosine Conjugates to Yeast

yeast strain	minimum inhibitory concn, μg/mL[a] pH 7.2 5-FC	conjugate I[b]	conjugate II[b]	pH 5.0 5-FC	conjugate I[b]	conjugate II[b]
C. albicans WD18-4	1.25	1.25	1.25	0.63	1.25	1.25
C. albicans I-V	1.25	1.25	1.25	1.25	2.5	2.5
C. krusei I-T	10.0	10.0	10.0	2.5	5.0	5.0
S. cerevisiae ATCC 9763	0.32	0.32	0.32	0.08	0.63	0.32
S. cerevisiae Z1-2D				0.04	0.63	

[a] MIC values generally represent average of four independent determinations. [b] Conjugate I = N^4-(succinyl-L-alanyl-L-leucine)-5-fluorocytosine; conjugate II = N^4-(succinyl-L-leucyl-L-leucine)-5-fluorocytosine.

II). We found that when present in 10-fold molar excess 5-FOrt-Leu-Leu, Ort-Leu-Leu, and N^4-(Succ-Ala-Leu-OH)-5-FC inhibited $(Met)_3$ uptake by 30, 65, and 55% respectively. In contrast, conjugates of 5-fluoroorotic acid with amino acids (e.g., 5-FOrt-Leu) as well as various orotyl–peptide esters (e.g., Ort-Met-Met-OMe) did not inhibit $(Met)_3$ transport. Furthermore orotylglycylglycine did not prevent $(Met)_3$ uptake into *C. albicans* WD 18-4. In addition we found that the inhibition of $(Met)_3$ uptake by 5-FOrt-Leu-Leu is competitive as judged by analysis of a double-reciprocal plot (Figure 1); the fluorinated pyrimidine–peptide conjugate has a K_I of 5×10^{-4} M at pH 3.5. We conclude that the competition studies demonstrate that 5-FOrt-Leu-Leu, N^4-(Succ-Ala-Leu-OH)-5-FC, Ort-Leu-Leu, and $(Met)_3$ have affinity for the same transport system in *C. albicans*.

Toxicity of Peptide–Fluoropyrimidine Conjugates. The minimum inhibitory concentration of various peptide–fluoropyrimidine conjugates was evaluated using the microdilution assay of Fisher and Armstrong (see Experimental Section). We found that the relative toxicity of 5-fluorocytosine and 5-fluorocytosine–peptide conjugates against various strains of yeast was dependent on both the strain and the pH in the assay medium (Table III). Specifically, both N^4-(Succ-Leu-Leu-OH)-5-FC and N^4-(Succ-Ala-Leu-OH)-5-FC exhibited toxicities equivalent to the free drug when tested against *C. albicans* at either pH 7.2 or 5.0 (maximum difference 1 dilution). However, when tested against *S. cerevisiae* the conjugates showed toxicities equal to free 5-FC only at pH 7.2 and exhibited lower toxicities (three to four dilutions) when tested at pH 5.0. In light of the hydrolytic stabilities of the peptide conjugates at pH 7.2 and 5.0, these results are evidence that the peptide conjugates are not transported into this strain of *S. cerevisiae* but do enter *C. albicans*. In contrast to the results with 5-fluorocytosine–peptide conjugates, 5-FOrt-Leu and 5-FOrt-Leu-Leu were not toxic against either yeast or Chinese hamster ovary (CHOP) cells, despite the fact that 5-fluoroorotic acid is highly toxic to CHOP cells.

Inhibition of 5-Orotidine Phosphoribosyltransferase by 5-Fluoroorotic Acid and 5-Fluoroorotyl–Peptide Conjugates. 5-Fluoroorotic acid is expected to inhibit orotate phosphoribosyltransferase.[10] Furthermore, this pyrimidine analogue can be metabolized to fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthetase.[11] We found that *C. albicans* WD 18-4 did contain activity which converted [^{14}C]orotic acid to [^{14}C]OMP and that this activity was strongly inhibited by 5-fluoroorotic acid (Figure 2). However, at similar concentrations 5-FOrt-Leu-Leu did not inhibit the activity of this enzyme.

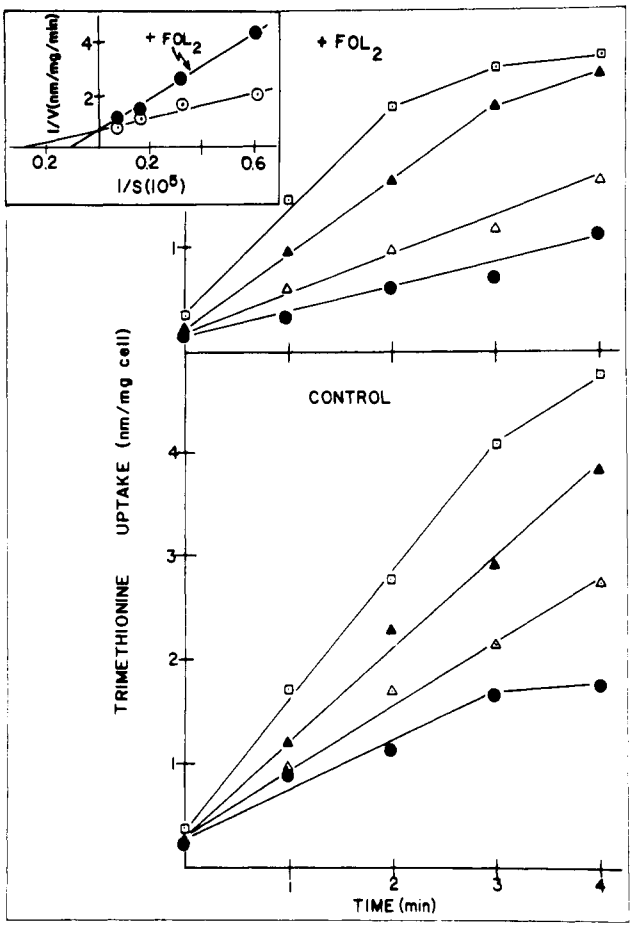

Figure 1. Uptake of radioactive trimethionine at different concentrations: (□) 12×10^{-5} M; (▲) 6×10^{-5} M; (△) 3×10^{-5} M; (●) 1.6×10^{-5} M. The upper panel labeled FOL_2 represents trimethionine uptake at different concentrations in the presence of 3×10^{-4} M 5-fluoroorotylleucylleucine. The insert in the top panel is a double-reciprocal analysis of the data.

Metabolism of 5-FOrt–Peptide Conjugates. The activity of cell extracts of *C. albicans* WD 18-4 against 5-FOrt–peptide conjugates was investigated. Incubation of 5-FOrt-Leu-Leu with cell extract (1 mg of protein/mL) from this yeast at pH 6.5 for 90 min did not result in any breakdown, even though under identical conditions $(Leu)_3$ is completely hydrolized to free leucine. In addition, using cell extract titered to pH 5.16 in order to activate a latent carboxypeptidase,[12] we found that 5-FOrt-Leu-Leu is partially degraded to 5-FOrt-Leu plus leucine (Figure 3). However, after a 90-min incubation, no leucylleucine or

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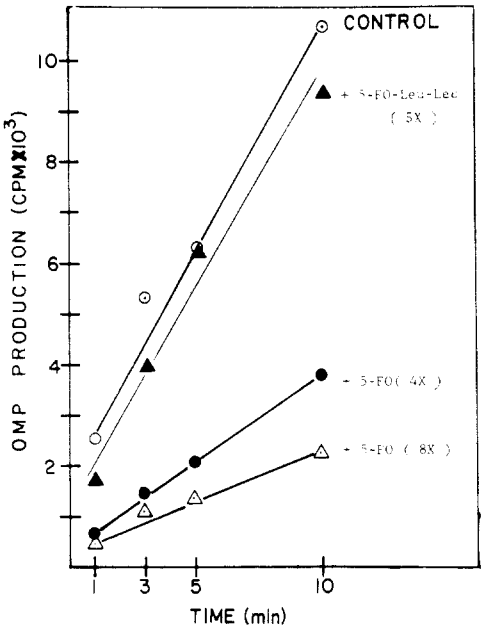

Figure 2. The synthesis of OMP (orotidine monophosphate) as an assay of orotate phosphoribosyltransferase is represented. Cell extracts were incubated with radioactive orotic acid (○) and at the time intervals indicated the reaction mixture was spotted on PEI-cellulose thin layers and chromatographed according to the Experimental Section to locate OMP. Competition experiments are represented by radioactive orotic acid with 5-fluoroorotylleucylleucine at fivefold excess (▲) and orotic acid with 5-fluoroorotic acid at a fourfold (●) and eightfold (△) excess.

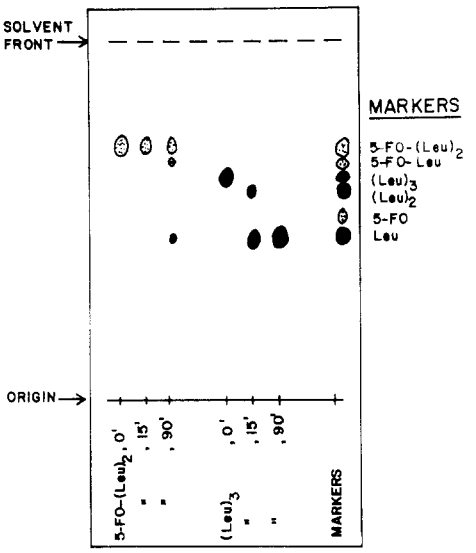

Figure 3. Thin-layer chromatogram of 5-fluoroorotylleucylleucine and trileucine metabolism by cell extracts. The extracts were incubated with the test compounds for the time periods indicated in the figure and then chromatographed on cellulose thin layers with 2-propanol/water (7:3) as the eluent. The markers are: leucine [Leu], 5-fluoroorotic acid [5-FO], leucylleucine [$(Leu)_2$], trileucine [$(Leu)_3$], 5-fluoroorotylleucine [5-FO-Leu], and 5-fluoroorotylleucylleucine [5-FO$(Leu)_2$]. The amino acids were located by ninhydrin staining and the fluoroorotyl-containing compounds by quenching of ultraviolet light.

free 5-fluoroorotic acid was obtained. Identical results were observed with extracts of CHOP cells. Finally, even incubation of 5-fluoroorotyl-L-leucine with pure renal hog acylase I for 2 days at pH 7.4 and 25 °C failed to liberate free 5-fluoroorotic acid.

Discussion

The results presented in this paper provide strong evidence that conjugates of pyrimidines and peptides can enter *C. albicans* through the peptide transport system. This conclusion is supported by the finding that Ort-Leu-Leu, 5-FOrt-Leu-Leu, and N^4-(Succ-Ala-Leu-OH)-5-FC all compete with trimethionine for entry into *C. albicans* WD 18-4 (Table II). Competition is competitive (Figure 1), and the pattern of competition which is observed is completely consistent with the structural requirements of the peptide transport system in this yeast in that neither amino acid conjugates nor peptide derivatives with protected carboxyl termini compete with peptide transport and that hydrophobic peptides are better competitors than those containing polar residues.[8]

In the case of conjugates of peptides with 5-fluorocytosine, entry into *Candida* is supported by evaluation of minimum inhibitory concentrations at different pH. At both pH 7.2 and 5.0, N^4-(Succ-Ala-Leu-OH)-5-FC and N^4-(Succ-Leu-Leu-OH)-5-FC were as toxic as free 5-fluorocytosine against three strains of *C. albicans*. In contrast, when tested against *S. cerevisiae* (Table III) at pH 5.0, these conjugates were significantly less active than 5-fluorocytosine. The lower toxicities against *S. cerevisiae* at pH 5.0 are consistent with the requirements for a free α-amine found for the peptide transport system in certain strains of *S. cerevisiae* (including *S. cerevisiae* Z1-2D)[13] and the increased hydrolytic stability of the conjugates in acidic media. The fact that the conjugates were less toxic against the two strains of *S. cerevisiae* suggest that they cannot enter these yeast cells and thus do not inhibit growth. The toxicity against *C. albicans* is therefore evidence that the peptide–5-fluorocytosine conjugate does enter this yeast and is metabolized to free 5-FC, which kills the cell. This entry occurs in *C. albicans* because this yeast (as demonstrated in *C. albicans* WD 18-4) can transport N-α-acylated peptides.

In contrast to results with *C. albicans* WD 18-4, N^4-(Succ-Ala-Leu-OH)-5-FC at 10-fold molar excess did not inhibit $(Met)_3$ uptake into *S. cerevisiae* Z1-2D. This finding supports our conclusion that the 5-fluorocytosine–peptide conjugates do not enter *S. cerevisiae*.

5-Fluoroorotic acid is not toxic to *C. albicans* WD 18-4 despite the fact that it is a strong inhibitor of 5-orotidine phosphoribosyltransferase activity in this yeast (Figure 2). We believe that the lack of toxicity of 5-FOrt is due to its failure to enter the cell, since we were unable to detect any uptake of radioactive 5-FOrt into yeast under conditions where 5-FC, amino acids, orotic acid, and peptides were readily transported. In an attempt to circumvent this permeability problem, we conjugated 5-FOrt to a number of peptides and examined their biological activity and their ability to compete with peptide transport. Although 5-FOrt-Leu-Leu competitively inhibited $(Met)_3$ uptake in *C. albicans* WD 18-4 (Figure 1), it did not kill this yeast. The failure of this conjugate to elicit a biological response could have resulted from its failure to enter the cell or from its failure to be metabolized to 5-fluoroorotic acid in the cytoplasm. Since our studies show that the amide bond linking 5-fluoroorotic acid to amino acids and peptides is stable to both enzymatic and hydrolytic breakdown, we speculate that 5-FOrt-Leu-Leu does enter the yeast but is not cleaved to free 5-fluoroorotic acid.

In conclusion, our findings represent the first examples of peptide–conjugates which can carry pyrimidine analogues into a eukaryotic cell. Thus, given the ability to design linkages which can be specifically cleaved by in-

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tracellular enzymes in *C. albicans*, peptides would be suitable carriers for drugs into this pathogenic yeast.

Experimental Section

Chemical Methods. Melting points were determined on a Hoover capillary melting point apparatus and are uncorrected. UV and NMR spectra were run on Cary 118C and JEOL MH-100 spectrometers, respectively. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and are within ±0.3% of calculated values. 5-Fluorocytosine was a generous gift of Dr. W. E. Scott of Hoffmann-La Roche, Nutley, N.J.

Synthetic Methods. The conjugation of various amino acid and peptide esters with orotic acid or 5-fluoroorotic acid was carried out using *N*-hydroxysuccinimide ester (method A) or 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) as the coupling agent (method B). All compounds subjected to microbiological evaluation gave the expected NMR resonances, absorbed in the appropriate regions of the IR and UV spectrum, and had correct elemental analyses. Detailed procedures are presented below for representative compounds.

Orotic Acid *N*-Hydroxysuccinimide Ester. Dicyclohexylcarbodiimide (11.3 g, 0.055 mol) was added to a solution of orotic acid (7.8 g, 0.05 mol) and *N*-hydroxysuccinimide (5.8 g, 0.05 mol) in 30 mL of dry Me_2SO. The mixture was stirred overnight at room temperature and the precipitate, dicyclohexylurea, was separated by filtration. Dry Me_2SO (15 mL) was used to wash the dicyclohexylurea, and the combined filtrate was slowly added to a 150-mL solution of 2-propanol–petroleum ether (1:6). After 2 h, the crystals which formed were isolated by filtration. Recrystallization from 2-propanol–ether gave 6.8 g (54% yield) of a white solid: mp 186–187 °C; NMR ($CDCl_3$) δ 3.0 (4 H, s, 2 × CH_2), 5.8 (1 H, s, 5-CH), 12.0 (2 H, br s, 2 × NH). This product was used in subsequent syntheses without further purification.

Orotyl-L-leucyl-L-leucine Methyl Ester. Method A. Orotic acid *N*-hydroxysuccinimide ester (0.3 g, 0.0012 mol) and leucylleucine methyl ester hydrochloride (0.35 g, 0.0012 mol) were dissolved in 20 mL of THF at room temperature. *N*-Methylmorpholine (0.12 g, 0.0012 mol) was added and the mixture was stirred at room temperature for 6 h. It was then diluted with 50 mL of ethyl acetate, the organic layer was washed with three 20-mL portions of 10% citric acid, three 20-mL portions of 5% sodium bicarbonate, and two 20-mL portions of distilled water and dried over anhydrous $MgSO_4$, and the solvent was removed under reduced pressure. The resulting oil was solidified by trituration with petroleum ether and recrystallized from methanol/ether (1:5) to give 0.12 g (25%) of a white crystalline product, mp 218 °C, which was homogeneous on silica thin layers: TLC R_f 0.3 (ethyl acetate), 0.71 (ethyl acetate/methanol, 2:1); $[\alpha]^{25}_D$ −32.27° (*c* 0.38, EtOH); NMR (Me_2SO-d_6) δ 0.8–1.0 (12 H, m, δ-Me), 1.4–1.9 (6 H, m, γ-CH, β-CH_2), 3.6 (3 H, s, $-OCH_3$), 4.1–4.6 (2 H, m, α-CH), 6.2 (1 H, s, 5-CH), 8.2 (1 H, d, NH), 8.7 (1 H, d, NH), 10.5 (1 H, s, NH), 11 (1 H, s, NH). Anal. ($C_{18}H_{28}N_4O_6 \cdot 0.5H_2O$) C, H, N.

Method B. A solution of orotic acid (1.56 g, 0.01 mol), leucylleucine methyl ester hydrochloride (2.94 g, 0.01 mol), *N*-methylmorpholine (1.01 g, 0.01 mol), and 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (2.47 g, 0.01 mol) in 25 mL of dry DMF was stirred at 50–55 °C overnight. After removal of the solvent in vacuo, the residue was washed three times with water (20 mL). It was solidified by trituration with petroleum ether and recrystallized from methanol/ether (1:5) to give 3.36 g (85%) of a white crystalline product which had properties identical with those described above.

5-Fluoroorotic Acid. 5-Fluoroorotic acid was synthesized using the procedure of Alam et al.[14] as modified below. *S*-Methylisothiouronium sulfate (5.56 g, 0.04 mol) was added to a solution of sodium diethylfluoroxaloacetate (18.24 g, 0.08 mol) and sodium ethoxide (2.85 g, 0.042 mol) in 150 mL of ethanol. The mixture was heated under reflux for 2 h, cooled, and evaporated to dryness under reduced pressure. The residue was taken up in 60 mL of ice-cold water, and the solution was extracted with ether (3 × 30 mL). The aqueous solution was acidified with 2 N hydrochloric acid to give 2.32 g (24.9%) of a precipitate, which was recrystallized from toluene to give 2.0 g of ethyl 2-(methylmercapto)-4-hydroxy-5-fluoro-6-pyrimidinecarboxylate, mp 184–185 °C. The pyrimidinecarboxylate (1.8 g) in concentrated hydrochloric acid (18 mL) was heated to reflux for 4 h and cooled in ice, and the crystalline solid was filtered and recrystallized from ethanol to yield 0.65 g (48.4%) of 5-fluoroorotic acid, mp 260–262 °C.

5-Fluoroorotyl-L-leucyl-L-leucine *tert*-Butyl Ester. A solution of 5-fluoroorotic acid (0.517 g, 0.003 mol), EEDQ (0.93 g, 0.00375 mol), HCl·Leu-Leu-O-*t*-Bu (1.25 g, 0.00375 mol), and *N*-methylmorpholine (0.38 g, 0.00375 mol) in 25 mL of DMF was stirred at 50–55 °C for 36 h. After removing the solvent under reduced pressure, the residue was dissolved in 25 mL of ethanol and poured into 200 mL of 1 N HCl. The precipitate was filtered and dried to give 1.36 g (98%) of a crude product which was further purified using high-performance LC on silica gel (E. Merck, size c) with cyclohexane/ethyl ether (1:2) as the eluent. The purified product, mp 152 °C, gave a single UV-positive spot on silica gel: TLC R_f 0.35 (ethyl acetate), 0.76 (ethyl acetate/MeOH, 2:1); $[\alpha]^{25}_D$ −25.4° (*c* 0.5, EtOH); NMR (Me_2SO-d_6) δ 0.8–1.0 (12 H, m, δ-CH_3), 1.5 (9 H, s, *t*-Bu), 1.2–1.8 (6 H, m, γ-CH, β-CH_2), 4.0–4.2 (1 H, m, α-CH), 4.2–4.5 (1 H, m, α-CH), 8.05 (1 H, d, NH), 8.5 (1 H, d, NH), 10.3 (1 H, s, NH), 11 (1 H, s, NH). Anal. ($C_{21}H_{33}N_4O_6F$) C, H, N, F.

5-Fluoroorotyl-L-leucyl-L-leucine. 5-Fluoroorotyl-L-leucyl-L-leucine *tert*-butyl ester (0.5 g, 0.0011 mol) was dissolved in 6 mL of 1 N HCl in acetic acid. The solution was stirred at room temperature for 2 h, after which HCl was removed in vacuo. Acetic acid was removed by freeze-drying and the residue was washed with anhydrous ether to give 0.42 g (95%) of a white solid, which was further purified by high-performance LC on silica gel (E. Merck, size c) using ethyl acetate/MeOH (2:1) as the eluent. The purified product, mp 201 °C, gave a single UV-positive spot on cellulose thin layers: TLC R_f (2-propanol/H_2O, 7:3) 0.75; $[\alpha]^{25}_D$ 12.0° (*c* 0.11, H_2O); NMR (Me_2SO-d_6) δ 0.9 (12 H, c, δ-CH_3), 1.2–1.8 (6 H, m, γ-CH, β-CH_2), 4.1–4.4 (2 H, m, α-CH), 8.1 (1 H, d, NH), 8.6 (1 H, d, NH). Anal. ($C_{17}H_{25}N_4O_6F$) C, H, N, F.

Kinetics. A solution of yeast nitrogen base (0.67%, Difco), including 3% dextrose and adjusted to the desired pH, was placed into thermally regulated reference and sample cells at 37 °C. The test compounds were dissolved in Me_2SO and injected into the sample cell so as to yield a final concentration of ca. 5×10^{-5} M. The absorbance at 305 nm was then recorded as a function of time. Compounds with $t_{1/2} > 5$ h were incubated in solutions from which portions were removed at appropriate intervals, and the absorbance was measured.

Biological Evaluation. The compounds were tested for anti-yeast activity by the method of Fisher and Armstrong[15] as previously described.[7] Briefly, yeast nitrogen base (0.67%, Difco) including 3% dextrose and 0.004% Bromthymol blue was adjusted to pH 7.2 or 5.0 and filter sterilized. Twofold serial dilutions of the test compounds in concentrations ranging from 1.28 mg/mL to 0.04 μg/mL were prepared in the growth medium and placed in flat-bottomed microtiter trays. Each well was inoculated with a 50-μL drop of the yeast suspension in growth medium and the test plate was incubated for 48 h at 37 °C. The minimum inhibitory concentration was read as the lowest concentration in which no color change in Bromthymol blue, from blue to yellow or light green, was detectable. The test organisms used were *Saccharomyces cerevisiae* 9763, the yeast used as the test organism for the 5-fluorocytosine assay in clinical laboratories; *Saccharomyces cerevisiae* Z1-2D, a strain known to have a functional peptide transport system;[13] *Candida albicans* 1-V, a clinical isolate received from the Center for Disease Control, Atlanta, Ga.; *Candida albicans* WD 18-4, the strain used in previous studies[7] and known to possess a peptide transport system; and *Candida krusei* 1-T, a *Candida* species with less pathogenic potential than *Candida albicans*.

Transport Assays. A. Growth of Cells. Strain *C. albicans* WD 18-4 was used for all transport assays. The conditions employed were identical with those described by Logan, Becker, and

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Naider.[8] Briefly, cells were grown in a modified Vogel's-N-medium, with isoleucine (6.56 mg/mL) replacing NH_4NO_3 as the nitrogen source. Starter cultures were grown at 37 °C with vigorous aeration for 18–24 h. This culture was inoculated into fresh medium contained in a 500-mL side-arm flask. Cells were then grown for 6–8 h (40–60 Klett units) as above. Growth was followed as an increase in turbidity at 400–420 nm (blue filter) using a Klett–Summerson photoelectric colorimeter.

B. Uptake of Met-Met-[^{14}C]Met. Cells were harvested by filtration using a Millipore apparatus (Millipore Corp., Bedford, Mass.) fitted with HAWP filters (Millipore Corp.) of 47 mm, 0.45-μm pore size, washed twice with cold distilled water, and resuspended in cold distilled H_2O such that a 1:10 dilution of the cells gave a reading of 30 (1.4 mg dry wt/mL) with the Klett colorimeter (blue filter). The cells were kept on ice until assayed. Prior to uptake, cells were incubated for 10 min at 37 °C with stirring and then added to an equal volume of a reaction mix at 37 °C which contained 2% glucose, citrate–phosphate buffer (pH 3.5, 0.012 M), and radioactive $(Met)_3$ (6.06×10^{-5} M, 1 μCi/μmol), synthesized as described previously.[16] At various time intervals, 500-μL portions were removed and filtered (Millipore Corp.; 25 mm, 0.45 μm) using a Millipore or a Yeda (Yeda Scientific Instruments, Rehovot, Israel) manifold. The filters were then washed twice with cold distilled H_2O, placed in Bray's solution,[17] and counted with a Searle IsoCap/300 6868 liquid scintillation system. For competition assays, the competing substrate was included in the reaction mix. Competitors were dissolved in distilled water or in dimethylformamide. In those experiments where dissolution in DMF was necessary, controls containing this cosolvent were included. In no case did DMF cause significant inhibition of $(Met)_3$ uptake.

C. Uptake of Orotic Acid and Fluoroorotic Acid. Transport assays for various pyrimidines were run essentially as described above for $(Met)_3$, except that the cells were equilibrated with buffer and glucose for 10 min at 37 °C prior to the addition of radioactive substrates. The final concentration of the orotic acids was 5×10^{-6} M, and the uptake conditions were optimized as to temperature, pH, and nitrogen source. Radioactive orotic acid, [6-^{14}C]orotic acid monohydrate (59 μCi/μmol), was purchased from Amersham Corp., Arlington Heights, Ill., and 5-fluoro[2-^{14}C]orotic acid (7.9 μCi/μmol) was obtained from ICN Pharmaceuticals, Irvine, Calif.

Assay of Orotate Phosphoribosyltransferase Activity. Cells grown for 19 h at 37 °C (100 mL) were harvested by centrifugation (100*g*), washed once with 0.9% cold NaCl, and resuspended in 20 mL of Tris buffer (pH 8, 0.5 M). The cell suspension and glass beads (40 g, Sigma Chemical Co., St. Louis, Mo., 75–100-μm diameter) were homogenized in a 50-mL size omnimixer stainless-steel cup (Ivan Sorvall Corp.). The suspension was kept on ice at top speed for 5 min, low speed for 1 min, and then an additional 10 min at top speed. The glass beads, unbroken cells, and cell debris were removed by centrifugation (1000*g*). The supernatant fluid was dialyzed against Tris buffer (pH 8, 0.05 M) at 4 °C overnight, and the resulting solution was used as a cell extract (CE). Protein determination on the CE was carried out by the Bio-Rad protein assay (Bio-Rad Labs, Richmond, Calif.). The protein content of the CE was 3.36 mg/mL. The CE was kept frozen (−20 °C) until just prior to use.

The assay was carried out according to Reyes.[18] Reaction mix containing, as final concentrations, phosphoribosyl pyrophosphate, 0.2 mM; $MgCl_2$, 4 mM; Tris-HCl (pH 8), 45 mM; and dithiothreitol, 0.7 mM, was prepared, and CE extract was added to a final protein concentration of 560 μg/mL. The reaction mix was incubated for 15 min at room temperature and at 37 °C for an additional 5 min; orotic acid, 15 mCi/mmol, was then added at a final concentration of 0.3 mM. Samples were removed at various time intervals and placed into 150 mM EDTA containing 5 mM aza-UMP to stop the enzymatic process.

The samples were spotted on PEI-cellulose plastic-backed sheets (Brinkman, Westbury, N.Y.), and each spot was then overlayed with a solution containing OMP, UMP, and orotic acid. TLC plates were developed using 0.2 M LiCl. After developing, spots were localized using ultraviolet light, and areas corresponding to the R_f values of OMP and UMP were cut out and placed in 0.5 mL of 0.1 M HCl, 0.2 M KCl contained in a minivial. These minivials were then shaken on a rotating shaker (200 rpm) for 30–60 min. Five milliliters of Bray's solution was added and vials were counted for radioactivity. In competition experiments, the competitor is added immediately prior to the orotic acid.

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