Anticandidal Activity of 5-Fluorocytosine–Peptide Conjugates

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An approach to the development of new anticandidal drugs is described that employs peptides as carriers of toxic agents into cells. 5-Fluorocytosine (5-FC) was chosen as a toxic agent with which to prepare 5-FC–peptide conjugates as models to test the carrier proposal. Model compounds were synthesized and then tested for antiyeast activity against *S. cerevisiae* 9763, *C. albicans* 1-V, *C. albicans* WD 18-4, and *C. krusei* 1-T. The 5-FC derivatives showed antiyeast activity comparable to 5-FC in all strains except *C. krusei* 1-T, in which case the compounds were less active. The solution stabilities of 5-FC conjugates at 37 °C were tested in the same growth medium used for susceptibility testing. The results indicated a range of stabilities where the half-life ($t_{1/2}$) = 0.3–17.6 h. These results and those obtained in the susceptibility testing suggest extracellular hydrolysis and indicate that the type of linkage used to conjugate 5-FC to peptides will not provide appropriate compounds to evaluate the peptide-carrier concept.

Candida albicans is one of the most commonly encountered microorganisms responsible for systemic infections in patients weakened by cancer, immunosuppressive therapy, and other debilitating conditions. The number of antimycotic agents available to effectively treat systemic fungal infections is limited to a few drugs, each of which has significant limitations.

We have proposed an approach to the development of new anticandidal drugs that exploits normal metabolic processes as a means of selectively delivering toxic agents into the cell:[1] A peptide acts as a discriminating drug carrier, selectively permeating the target cells. Once inside the cell normal metabolism results in the release of a substance toxic to the cell.

The molecular basis for cellular permeation of peptides has been studied in bacteria, yeast, and mammalian cells.[2-5] Peptides are ideally suited to act as carrier species because the chain length and side chain specificities of transport are quite broad.[2] Furthermore, since the structural requirements for transport differ for bacteria, yeast, and mammalian cells, peptide conjugates can in principle be designed so as to be selective for yeasts.

Peptides have been used to carry normally impermeable substances into *E. coli*[6] and *S. typhimurium*.[7] We have studied peptide transport in *S. cerevisiae*[8-10] and *C. albicans*,[11] and the results pertinent to anticandidal drug development are summarized as follows: (1) *Candida* like most bacteria and like *S. cerevisiae* can utilize dipeptides and oligopeptides as sources of amino acids. (2) *C. albicans* and *S. cerevisiae* can utilize certain N-acylated di- and tripeptides. This differs from *E. coli*[3] and mammalian gut[12] where peptides acylated at the amino terminus are not transported. (3) Methyl esters of tri-, tetra-, and pentapeptides can not enter *C. albicans*, although the corresponding free carboxylic acids are readily utilized as growth substrates. This is in contrast to bacteria where peptides with modified terminal carboxyl groups are accepted by the oligopeptide permease. (4) Peptides containing D residues are utilized by *Candida albicans*. Such peptides would be expected to be more resistant to serum peptidase hydrolysis and, therefore, potentially useful as in vivo carriers. In summary, drug design for the peptide carriers should include a free terminal carboxyl to permit uptake by *Candida* and also an acylated amino terminus to prevent entry into bacteria and perhaps mammalian tissue.

5-Fluorocytosine (5-FC), a clinically used drug, was selected for attachment to a peptide carrier. This drug was chosen because of its known toxicity to *C. albicans* and because the mechanisms of resistance to this compound have been studied in both *S. cerevisiae*[13,14] and *C. albicans*.[15-17]

We chose to form an amide bond between the N^4-amino group of the fluorinated pyrimidine and a carboxyl function of the peptide as the means of conjugating the peptide and drug. This type of linkage was expected to maximize the probability of recognition by the oligopeptide permease and was expected to be susceptible to enzymatic hydrolysis inside the cell. Since the peptide transport system in *Candida* requires a free terminal carboxyl function, the amine terminus of the peptide was succinylated, providing a carboxyl function to form the necessary amide bond. As an alternate approach, 5-fluorocytosine was conjugated to the γ-carboxyl of a glutamic acid residue, for use in the preparation of a peptide with a free terminal amine and carboxyl group.

In this report, we describe the synthesis of model compounds and the results of yeast susceptibility tests. In light of the fact that N^4-acylated cytosines are known to be sensitive to acid and base hydrolysis, it seemed prudent to determine the solution stability of these compounds in the media used for susceptibility testing prior to preparing final compounds. The results of these experiments are discussed with respect to the drug–carrier model.

Chemistry. The N^4-amino group of 5-FC is weakly basic ($pK_a' = 2.90$) and not easily acylated. Acid chlorides in pyridine or anhydrides of low-molecular-weight aliphatic and aromatic carboxylic acids, at reaction temperatures of >100 °C, have been used to prepare mono-N^4-acylated 5-fluorocytosines.[18] We did not succeed in preparing **11** (Scheme I) by reacting 5-FC with the acid chloride of β-carboxypropionyl-Gly-Gly-OBz in pyridine at 100 °C. As an alternative approach, we unsuccessfully sought to prepare N^4-(β-carboxypropionyl)-5-fluorocytosine by reacting 5-FC with succinic anhydride. Attempts to couple β-carboxypropionyl-Gly-Gly-OBz (**8**) to 5-FC with dicyclohexylcarbodimide also did not yield the desired products. We were, however, able to prepare **11** by coupling 5-FC and **8** using 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) in anhydrous DMF at 35–40 °C (Scheme I). The reaction conditions used in this method are mild and unlikely to induce racemization with its accompanying potential for losing biological activity. EEDQ reacts by a mechanism[19] that allows slow release of a mixed anhydride and is, therefore, well suited to the poor solubility properties of 5-FC at low temperatures. We used this procedure to prepare N^4-(Boc-glutamyl-α-OBz)-5-FC (**18**), a compound which was also synthesized

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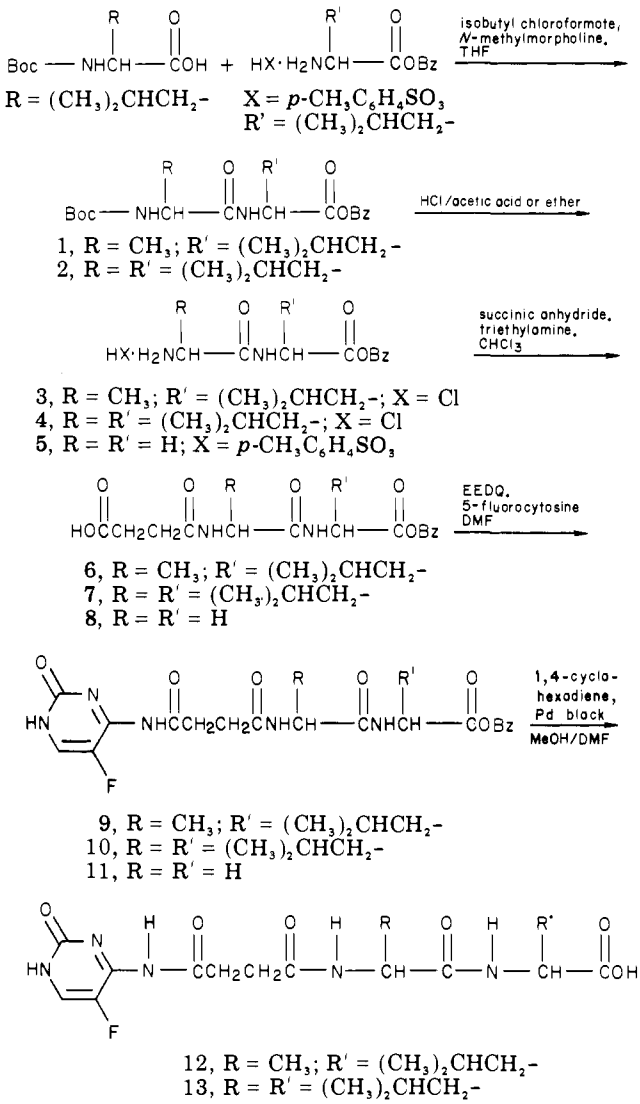
Scheme I. Synthesis of 5-FC-Peptide Conjugates



by the anhydride method[18] albeit in low (22%) yield. The products of these two synthetic approaches were judged identical by IR, NMR, TLC, and UV analysis. We subsequently prepared a series of peptide–5-FC conjugates in good yield by the EEDQ method. Peptide benzyl ester–5-FC conjugates prepared by the EEDQ method were deblocked by transfer hydrogenation with palladium black and 1,4-cyclohexadiene in a solution of either anhydrous dimethylformamide or methanol–dimethylformamide.

tert-Butoxycarbonyl-substituted dipeptide benzyl esters were prepared from the appropriately blocked amino acids by the mixed anhydride coupling techniques. Boc groups were removed with 1 N HCl in acetic acid, producing hydrochlorides that were reacted with succinic anhydride and triethylamine in chloroform to form β-carboxypropionyl-substituted peptide benzyl esters.

N^4-Ac- (**15**), N^4-(Cbz-Gly)- (**16**), and N^4-(Boc-Ala-Gly)-5-FC (**17**) were prepared by reacting the appropriate *p*-nitrophenyl esters with 5-FC in dimethylformamide using 1-hydroxybenzotriazole as a catalyst. Details of this procedure will be published elsewhere.

Experimental Section

Chemical Methods. Melting points were determined on a Hoover capillary melting point apparatus and are uncorrected. UV and IR spectral data were obtained on Cary 118 and Beckman 4260 spectrophotometers, respectively. NMR spectra were run on a JEOL MH-100 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and are within ±0.3% of calculated values. Glycylglycine benzyl ester *p*-tosylate, L-leucine benzyl ester *p*-tosylate, and *tert*-butoxycarbonyl-L-leucine and *tert*-butoxycarbonyl-L-alanine were purchased from Bachem, Inc., Torrance, Calif. 5-Fluorocytosine was a generous gift of Dr. W. E. Scott of Hoffmann-La Roche, Nutley, N.J.

Synthetic Methods. *tert*-Butyloxycarbonyl-L-alanyl-L-leucine Benzyl Ester (1). *tert*-Butoxycarbonyl-L-alanine (4.39 g, 0.023 mol) was dissolved in tetrahydrofuran (125 mL) and the resulting solution was cooled to −20 °C with a dry ice–carbon tetrachloride bath. *N*-Methylmorpholine (2.35 g, 0.023 mol) was added, followed by isobutyl chloroformate (3.16 g, 0.023 mol). The resulting mixture was stirred for 3 min at −20 °C, at which time a solution of L-leucine benzyl ester *p*-tosylate (2.44 g, 0.024 mol) and *N*-methylmorpholine (2.44 g, 0.024 mol) in dimethylformamide was added. The reaction mixture was stirred for 1 h at −20 °C and then allowed to warm slowly to room temperature, at which time it was diluted with ethyl acetate. The organic mixture was washed with 10% citric acid (three times), distilled water (one time), 5% sodium bicarbonate (three times), and saturated sodium chloride (one time). The remaining organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure, yielding an oily residue (7.5 g). TLC analysis on silica gel (ethyl acetate–petroleum ether, 1:3) indicated two minor impurities. Attempts to induce crystallization were unsuccessful. The compound was purified using high-performance liquid chromatography on a prepacked silica column (E. M. Merck-Darmstadt, size C) equilibrated with petroleum ether. The product (7.5 g) was applied onto the column in methylene chloride (10 mL) and then washed-in with additional (1 mL) methylene chloride. The material was eluted with petroleum ether (200 mL), petroleum ether–ethyl acetate (3:1, 300 mL), and then petroleum ether–ethyl acetate (1:2). Combination of fractions and removal of solvent yielded an oil (6.8 g) that was homogeneous on silica thin layers [petroleum ether, petroleum ether–ethyl acetate (3:1), ethyl acetate–petroleum ether (2:1)] and analytically pure: NMR δ ($CDCl_3$) 0.9 (6 H, m, δ-Me), 1.34 (3 H, d, β-Me), 1.45 (9 H, s, Me), 1.64 (3 H, complex, CCH_2 and CCH), 4.04–4.40 (1 H, m, α-CH), 4.56–4.88 (1 H, m, α-CH), 5.20 (1 H, d, partially overlapped, NH), 5.24 (2 H, s, benzyl), 6.76 (1 H, d, NH), 7.45 (5 H, s, aryl). Anal. ($C_{21}H_{32}N_2O_5$) C, H, N.

β-Carboxypropionyl-L-alanyl-L-leucine Benzyl Ester (6). A solution of 1 (6.7 g, 0.017 mol) in 1 N HCl in acetic acid was stirred for 1 h at room temperature, at which time excess hydrogen chloride was removed in vacuo. The resulting solution was lyophilized, yielding an oil. L-Alanyl-L-leucine benzyl ester hydrochloride[9] solidified (4.5 g) on trituration with hexane and was homogeneous on thin layers of silica gel (chloroform–methanol, 2:1). The compound was used without further purification. Succinic anhydride (1.61 g, 0.016 mol) was allowed to react overnight with a solution of **3** (0.012 mol) and triethylamine (1.62 g, 0.016 mol) in chloroform (100 mL) at room temperature. The chloroform solution was washed with distilled water and then extracted with 5% sodium bicarbonate. The aqueous phase was then acidified to pH 3.0 with 10% citric acid and extracted with chloroform. Removal of the solvent yielded an oil that solidified when triturated with petroleum ether–ethyl acetate. This solid (3.7 g, 81%) was homogeneous on silica thin layers [chloroform–methanol (2:1) and isopropyl alcohol–water (7:3)]. Recrystallization from ethyl acetate yielded an analytical sample: mp 88–89 °C; NMR δ ($CDCl_3$) 0.96 (6 H, m, δ-Me), 1.24 (3 H, d, β-Me), 1.42–1.76 (3 H, m, CCH_2 and CCH), 2.36–2.80 (4 H, m, 2 × $CH_2C{=}O$), 4.4–4.8 (2 H, m, α-CH), 5.16 (2 H, s, benzyl), 7.18 (2 H, d, NH), 7.84 (5 H, s, aryl), 9.18 (1 H, br s, COOH); IR ν_{max} (KBr) 3290, 1746, 1730, 1645 and 1550 cm^{-1}. Anal. ($C_{20}H_{28}N_2O_6$) C, H, N.

***tert*-Butoxycarbonyl-L-leucyl-L-leucine Benzyl Ester (2).** This compound was prepared from *tert*-butoxycarbonyl-L-leucine monohydrate (10.0 g, 0.04 mol) and L-leucine benzyl ester *p*-tosylate (17.7 g, 0.045 mol) by the mixed anhydride procedure as described for the preparation of 1. The workup yielded an oil that crystallized on standing (16.1 g, 92%), mp 92–93 °C (lit.[20] 92–92.5 °C).

β-Carboxypropionyl-L-leucyl-L-leucine Benzyl Ester (7). Anhydrous hydrogen chloride was bubbled through a solution of

2 (8.0 g, 0.0184 mol) in anhydrous ether (90 mL) until the solution was saturated and then allowed to continue for an additional hour. L-Leucyl-L-leucine benzyl ester hydrochloride (4) crystallized from the ether solution and was collected by filtration and washed with anhydrous ether. The white crystalline product (6.8 g, >99%) was homogeneous on thin layers of silica gel [chloroform–methanol (2:1)] and was used without further purification. 4 (6.8 g, 0.0184 mol) was added to a mixture of succinic anhydride (2.22 g, 0.022 mol) and triethylamine (2.22 g, 0.022 mol) in chloroform (100 mL) and allowed to react overnight at 25 °C. The chloroform solution was then washed with water (three times), 10% citric acid (three times), and saturated sodium chloride. The solution was dried ($MgSO_4$) and the solvent removed in vacuo, leaving an oily residue (6.6 g) that crystallized on trituration with petroleum ether (5.9 g, 74%), mp 95–96 °C. The product was homogeneous on a silica thin-layer chromatogram [chloroform–methanol–acetic acid (95:15:3)]: NMR δ (Me_2SO-d_6) 0.52–1.02 (12 H, m, δ-CH_3), 1.02–1.82 (6 H, m, β-CH_2, γ-CH), 2.22–2.42 (4 H, m, α-CH_2), 4.12–4.50 (2 H, m, α-CH), 5.10 (2 H, s, benzyl), 7.40 (5 H, s, aryl), 8.02 (1 H, d, J = 7.5 Hz, NH), 8.50 (1 H, d, J = 8.0 Hz, NH); IR ν_{max} (KBr) 3310, 1742, 1725, 1700, 1640, 1540 cm^{-1}. Anal. ($C_{23}H_{34}N_2O_6$) C, H, N.

δ-Carboxypropionylglycylglycine Benzyl Ester (8). A solution of succinic anhydride (4.44 g, 0.044 mol) in chloroform (400 mL) was charged with glycylglycine benzyl ester p-tosylate (5; 15.76 g, 0.04 mol) and triethylamine (4.44 g, 0.044 mol). The resulting mixture was stirred overnight, and the product which crystallized from solution was collected by filtration and washed with excess chloroform. The white crystalline product (11.8 g, 92%), mp 155–156 °C, showed only one spot on silica thin-layer chromatograms [chloroform–methanol–acetic acid (95:15:3)] and on cellulose thin layers [isopropyl alcohol–water (7:3)]. Recrystallization from ethanol yielded an analytical sample: NMR δ (Me_2SO-d_6) 2.32 (4 H, s, 2 CH_2C=O), 3.75 (2 H, d, α-CH_2), 3.92 (2 H, d, α-CH_2), 5.11 (2 H, s, benzyl), 7.32 (5 H, s, aryl), 8.06–8.38 (2 H, overlapping triplets, 2 NH); IR ν_{max} (KBr) 3280, 1748, 1728, 1642, 1203 and 1214 cm^{-1}. Anal. ($C_{15}H_{18}N_2O_6$) C, H, N.

5-Fluoro-4-(*N*-succinamoyl-L-leucyl-L-leucine benzyl ester)-2(1*H*)-pyrimidone (10). Both 7 (5.8 g, 0.013 mol) and 5-fluorocytosine (1.29 g, 0.01 mol) were suspended in anhydrous dimethylformamide (30 mL) at 40 °C. EEDQ (3.3 g, 0.013 mol) was then added, and the reaction mixture was stirred overnight while maintaining the bath temperature between 35 and 40 °C. The solution was concentrated in vacuo to an oily solid which was free of dimethylformamide. This residue was then stirred in a large excess of anhydrous ether. A white solid (3.9 g, 72%) was separated from this mixture by filtration. Thin-layer chromatograms on silica gel [chloroform–methanol–acetic acid (95:15:3)] indicated a trace impurity with an R_f identical with that of 5-fluorocytosine. Recrystallization from ethanol provided an analytical sample (mp 184 °C) that was homogeneous on silica and cellulose [isopropyl alcohol–water (7:3)] layers: NMR δ (Me_2SO-d_6) 0.98 (12 H, m, 4 δ-CH_3), 1.20–1.84 (6 H, m, 2 δ-CH_2 and 2 γ-CH), 2.50 (2 H, br t, overlapping solvent peak, CH_2C=O), 2.84 (2 H, br t, CH_2C=O), 4.28–4.68 (2 H, m, 2 α-CH), 5.27 (2 H, s, benzyl), 7.62 (5 H, s, aryl), 8.28 (1 H, d, NH), 8.38 (1 H, d, C=CH), 8.52 (1 H, d, NH); UV λ_{max} (EtOH) 349 nm (ϵ 5800), 292.5 (3200), 244 (8700). Anal. ($C_{27}H_{36}FN_5O_6$) C, H, F, N.

5-Fluoro-4-(*N*-succinamoyl-L-alanyl-L-leucine benzyl ester)-2(1*H*)-pyrimidone (9). 6 (1.71 g, 0.0045 mol) and 5-fluorocytosine (0.39 g, 0.003 mol) were suspended in 15 mL of anhydrous dimethylformamide at 40 °C and treated with EEDQ (1.12 g, 0.0045 mol) as described for 10 (yield, 53%). Recrystallization from absolute ethanol yielded an analytical sample: mp 191–196 °C; NMR δ (Me_2SO-d_6) 0.72–1.0 (6 H, m, δ-CH_3), 1.15 (3 H, d, β-CH_3), 1.4–1.8 (3 H, complex, β-CH_2 and γ-CH), 2.2–2.88 (4 H, complex, CH_2CH_2 and solvent), 4.08–4.52 (2 H, m, α-CH), 5.06 (2 H, s, benzyl), 7.38 (5 H, s, aryl), 8.0–8.40 (3 H, complex, Ala-NH, Leu-NH and CH=C); IR ν_{max} (KBr) 3290, 1737, 1548, 1510, 1460, 1195 cm^{-1}; UV λ_{max} (EtOH) 348 nm (ϵ 5100), 285 (3300), 241.5 (8600). Anal. ($C_{24}H_{30}FN_5O_6$) C, H, F, N.

5-Fluoro-4-(*N*-succinamoylglycylglycine benzyl ester)-2(1*H*)-pyrimidone (11). β-Carboxypropionylglycylglycine benzyl ester (3.75 g, 0.012 mol) and 5-fluorocytosine (0.75 g, 0.006 mol) were suspended in 42 mL of anhydrous dimethylformamide at 40 °C. EEDQ (2.88 g, 0.012 mol) was added, and the resulting mixture was stirred overnight at 40 °C. The mixture was then filtered and the residue washed with anhydrous ethyl acetate. The crystalline residue (1.4 g, 56%) was homogeneous on silica thin layers [chloroform–methanol (2:1) and isopropyl alcohol–water (7:3)]. The solvent was removed from the filtrate, yielding an oil that crystallized (0.45 g) on standing. Thin-layer analysis on silica indicated that these crystals were mainly product but contained a small impurity. The product was recrystallized from ethanol–water, yielding an analytically pure sample: mp 194–195 °C; NMR δ (Me_2SO-d_6) 2.32–2.90 (4 H, complex, CH_2CH_2 and solvent resonance), 3.72 (2 H, d, α-CH_2), 3.90 (2 H, d, α-CH_2), 5.13 (2 H, s, benzyl), 7.40 (5 H, s, aryl), 8.14 (1 H, d, J = 10.5 Hz, CH=CF), 8.20–8.44 (2 H, br t, 2 Gly-NH); IR ν_{max} (KBr) 1736, 1705, 1640, 1628, 1595, 1565 cm^{-1}; UV λ_{max} (H_2O, solubilized with 0.8% Me_2SO) 305 nm (log ϵ 3.76), 241 (4.04). Anal. ($C_{19}H_{20}FN_5O_6$) C, H, F, N.

N^4-γ-(*tert*-Butoxycarbonyl-L-glutamyl-α-benzyl ester)-5-fluorocytosine (18). Procedure A. *tert*-Butoxycarbonyl-L-glutamic acid α-benzyl ester (19; 9.8 g, 0.029 mol) and 5-fluorocytosine (2.5 g, 0.019 mol) was suspended in anhydrous dimethylformamide (150 mL) at 40 °C. EEDQ (7.2 g, 0.029 mol) was added and the reaction stirred overnight at 40 °C. Removal of the solvent in vacuo yielded an oil that crystallized on addition of anhydrous ether. The crude product (5.6 g, 65%) showed an impurity on thin-layer silica plates [chloroform–methanol–acetic acid (95:15:3)]. Recrystallization from tetrahydrofuran–H_2O yielded a homogeneous sample: yield 4.7 g (54%); mp 156–157 °C; NMR δ (Me_2SO-d_6) 1.36 (9 H, s, 3 CH_3), 1.62–2.32 (2 H, m, CH_2), 2.60 (2 H, complex, solvent peaks and CH_2C=O), 3.82–4.19 (1 H, m, α-CH), 5.19 (2 H, s, benzyl), 7.20 (1 H, d partially overlapping aryl, Glu-NH), 7.26 (5 H, s, aryl), 7.98 (1 H, d, HC=C); IR ν_{max} (KBr) 3380, 1740, 1690, 1665, 1525, 1470, 1208 cm^{-1}; UV λ_{max} (95% EtOH) 347 nm (log ϵ 3.79), 313 (3.73), 243.5 (4.02), 209 (4.24). Anal. ($C_{21}H_{25}FN_4O_6$) C, H, F, N.

Procedure B. A solution of 19 (10.11 g, 0.03 mol) in 50 mL of chloroform was treated with dicyclohexylcarbodiimide (3.1 g, 0.015 mol). The reaction mixture was stirred for 15 h at room temperature, at which time it was filtered, and the filtrate was concentrated to dryness in vacuo. The residue was then redissolved in ethyl acetate and cooled for several hours. The resulting small precipitate of dicyclohexylurea was collected by filtration and the filtrate was again concentrated in vacuo. This process was repeated until all the dicyclohexylurea was removed. The oily residue was then crystallized (5.7 g, 58%) from ethyl acetate–hexane, mp 91–93 °C. The anhydride product 14 which was homogeneous on silica thin layers (ethyl acetate) showed an R_f different from 18 and gave the expected IR bands: NMR δ ($CDCl_3$) 1.44 (9 H, s, 3 CH_3), 1.56–2.72 (4 H, complex, CH_2C=O and CH_2), 4.24–4.64 (1 H, m, α-CH), 5.30 (2 H, s, benzyl), 5.30–5.48 (1 H, NH, partially overlapping benzyl), 7.48 (5 H, s, aryl); IR ν_{max} (KBr) 3350, 1818, 1750, 1725, 1690, 1510, 1252, 1065, 1055 cm^{-1}.

14 (5.2 g, 0.008 mol) was added to a solution of 5-fluorocytosine (0.52 g, 0.004 mol) in anhydrous pyridine at 100 °C and allowed to react at this temperature for 5 h. The solution was cooled and the solvent concentrated in vacuo, yielding an oily solid that solidified (1.0 g) on trituration with ether. Thin-layer analysis on silica gel [chloroform–methanol–acetic acid (95:15:3)] and an NMR spectrum indicated contamination with 5-fluorocytosine. The solid was then suspended in chloroform, stirred for 2 h, and then filtered. The filtrate was concentrated in vacuo to a glassy solid that crystallized from tetrahydrofuran–water, yielding a homogeneous product (0.39 g, 22%), mp 156–157 °C. This compound was identical in all respects with the product obtained in procedure A.

5-Fluoro-4-(*N*-succinamoyl-L-leucyl-L-leucine)-2(1*H*)-pyrimidone (13). Freshly prepared palladium black was added to a solution of 10 (0.4 g, 0.00072 mol) in anhydrous methanol–dimethylformamide (24 mL, 5:1). 1,4-Cyclohexadiene (1.36 mL, 0.00144 mol) was added to the mixture, and the reaction was stirred at room temperature for 2.5 h. The catalyst was then removed by filtration and the solvent removed in vacuo. A white solid formed when ether was added to the dried residue. The compound (0.32 g) was recrystallized from THF–water, yielding a product (0.077 g), mp 184–185 °C, that was homogeneous on silica thin layers [dichloromethane–methanol–acetic acid (95:15:3)]:

Table I. Results of Susceptibility Tests Using the Microdilution-Colorimetric Method of Fisher and Armstrong[a]

compd	$t_{1/2}$,[d] h	minimum inhibitory concn, μg/mL[b] *S. cerevisiae* 9763	*C. albicans* 1-V	*C. albicans* WD 18-4	*C. krusei* 1-T
5-fluorocytosine		0.31	0.31	5	5
9	1.1	0.31	0.62	5	20
10		0.31	0.62	2.5	20
11	0.3	0.62	0.62	5	20
12	1.7	0.31	0.31	5	10
13	2.9	0.31	0.31	5	10
7		80	80	>20[c]	320
6		160	80	>20[c]	320
8		320	320	>20[c]	320
succinic acid		640	320	>20[c]	320
N^4-Ac-5-FC (**15**)	17.6	0.31	2.5	5	40
18	10.0	1.25	2.5	2.5	20
N^4-(Boc-Ala-Gly)-5-FC (**17**)	2.3	0.62	0.62	1.25	20
N^4-(Cbz-Gly)-5-FC (**16**)		0.31	0.62	2.5	10
5-fluorouracil		1.25	2.5	>20[c]	2.5

[a] See ref 21. [b] μg/mL normalized to molar equivalents of 5-FC. [c] Highest level tested. [d] Half-lives measured in test medium at 37 °C.

NMR δ (Me_2SO-d_6) 0.84 (12 H, overlapping doublets, δ-CH_3), 1.28–1.68 (6 H, m, β-CH_2 and γ-CH), 2.52–2.84 (4 H, m, 2 CH_2C=O), 3.84–4.32 (2 H, m, 2 α-CH), 7.50–7.80 (3 H, complex, 2 Leu-NH, C=CH); UV λ_{max} (H_2O, pH 6.3, solubilized with 1% Me_2SO) 305 nm (ε 9300), 242 (17 950).

5-Fluoro-4-(*N*-succinamoyl-L-alanyl-L-leucine)-2-(1*H*)-pyrimidone (12). A solution of **9** (0.3 g, 0.00059 mol) in anhydrous dimethylformamide (10 mL) was treated with freshly prepared palladium black and 1,4-cyclohexadiene. The resulting mixture was stirred at room temperature for 2 h, filtered to remove the catalyst, and then concentrated in vacuo. On the addition of ether a solid formed (234 mg) and was subsequently collected by filtration. The white solid was stirred in anhydrous acetonitrile (50 mL) for 2 h, filtered, and washed with more acetonitrile and then ether. The dried product (193 mg, 79%) was homogeneous on silica thin layers [1-butanol–acetic acid–water (4:1:1), isopropyl alcohol–water (7:3)]: mp 180–181 °C; NMR δ (Me_2SO-d_6) 0.83 (6 H, overlapping doublets, δ-CH_3), 1.2 (3 H, d, β-CH_3), 1.38–1.72 (3 H, m, β-CH_2 and γ-CH), 2.28–2.88 (4 H, complex, CH_2CH_2 and solvent), 3.92–4.48 (2 H, complex, α-CH), 7.76–8.0 (3 H, complex, Ala-NH, Leu-NH and CH=C); UV λ_{max} (H_2O, pH 6.3, solubilized with 1% Me_2SO) 304 nm (ε 8400), 242.5 (16 500).

Kinetics. A solution of yeast nitrogen base (0.67%, Difco), including 3% dextrose and adjusted to pH 7.2 with 10% KOH, 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 (Table I). The compounds were tested for antiyeast activity by the method of Fisher and Armstrong.[21] Yeast nitrogen base (0.67%, Difco) including 3% dextrose and 0.004% Bromothymol blue was adjusted to pH 7.2 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. An extra well with the highest drug concentration was used as an organism-free control, and another well with no drug was used as a drug-free control. Each well, with the exception of the organism-free control, was inoculated with a 50-μL drop of the yeast suspension in growth medium. The innoculum was an overnight culture resuspended to a concentration of about 2×10^6 cells/mL. The test plate was incubated for 48 h at 37 °C and readings were made at 24 and 48 h. Bromthymol blue dye served as an indicator of growth as the growing yeast produces acid causing a color change from blue to yellow. Growth could also be assessed by visual inspection of cell pellets in the microtiter wells. The minimum inhibitory concentration was read as the lowest concentration in which no color change, 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; *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[11] and known to possess a peptide transport system; and *Candida krusei* 1-T, a *Candida* species with less pathogenic potential than *Candida albicans*.

Results and Discussion

The 5-FC–peptide conjugates were tested for antifungal activity against *Saccharomyces cerevisiae* 9763, *Candida albicans* WD 18-4, *Candida albicans* 1-V, and *Candida krusei* 1-T by the microdilution–colorimetric method of Fisher and Armstrong.[21] The results, which are summarized in Table I, represent the minimum inhibitory concentration (MIC) for total inhibition of fungal growth. To simplify interpretation, MIC values of 5-FC-containing compounds were adjusted so as to reflect molar equivalents of 5-FC. Readings were made at 24 and 48 h. No differences were noted for the two readings. The activities of the 5-FC derivatives tested were comparable to the parent compound (5-FC) against all strains tested, with the exception of *C. krusei*. The MIC values in most cases are either identical with 5-FC or reflect one dilution difference. In contrast, 5-FC-free fragments of the peptide–drug conjugates, such as succinylated peptide benzyl esters **6**–**8** and succinic acid, were inactive. Thus, the observed activities must result from one or more of the following possibilities: (a) The conjugate is transported into the cell and is itself toxic. (b) The compound is hydrolized subsequent to transport, releasing 5-FC which kills the cell. (c) The derivative is hydrolyzed prior to transport, releasing 5-FC which then permeates into the cell. The biological data do not allow for clear distinctions to be drawn between these possible modes of action. However, our results and those of other workers lead us to a tentative conclusion.

Previous studies[11] have established a functional peptide-transport system in *C. albicans* and also indicated that peptide esters would be excluded from this transport system. Thus, it seems unlikely that the 5-FC–peptide ester conjugates could enter the cell by the peptide-transport system. However, other routes of cell penetration and strain variation in the structural specificity of peptide transport cannot be ruled out. The possibility that 5-FC–peptides are hydrolized prior to cell uptake is supported by the results of solution stability tests.

The 5-FC derivatives **9**, **11**–**13**, **15**, **17**, and **18** were incubated at 37 °C in solutions of the organism-free yeast nitrogen base used for susceptibility testing. Hydrolysis

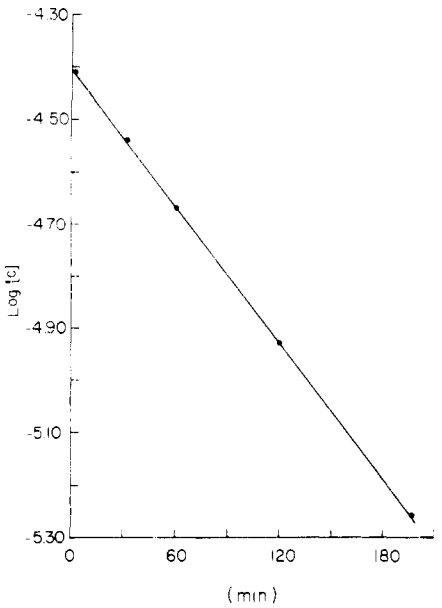


Figure 1. Decomposition of 5-fluoro-4-(*N*-succinamoylalanylleucine benzyl ester)-2(1*H*)-pyrimidone at 37 °C in yeast nitrogen base. The concentration of the acyl peptide conjugate was determined by measuring its absorbance at 305 nm in yeast nitrogen base (0.67%, Difco) supplemented with 3% dextrose and adjusted to pH 7.2 with 10% KOH.

was monitored by UV as a function of time. 5-FC, shown by TLC to be a major product of hydrolysis, has a characteristic absorption λ_{max} 277 nm in neutral aqueous solutions and like other cytosines with a free N^4-amino group differs from N^4-acylated cytosines which have characteristic absorptions in the 300-nm region.[22,23] Decreases in absorbance at 305 nm were used to monitor the hydrolysis of the acylated 5-FC derivatives. All compounds investigated showed first-order kinetics (Figure 1) and had half-lives ranging from 0.3 to 17.6 h (Table I). Of the compounds tested, N^4-Ac-5-FC and N^4-(Boc-Glu-α-OBz)-5-FC (**18**) were the most stable with $t_{1/2}$ values of 17.6 and 10.0 h, respectively. Peptide-5-FC conjugates **9** and **11** prepared with a succinic acid spacer group were less stable than **17**, which was formed by direct conjugation of the peptide terminal carboxyl with the N^4-amino group of 5-FC. All of the derivatives were quite labile, a result consistent with a mode of action involving hydrolysis and release of 5-FC prior to transport.

Based on biological studies, acylated 5-FC derivatives were reported[18] to release 5-FC. The mechanism and site of release, however, was not discussed. Our stability studies show that in most cases acylated 5-FC derivatives would be expected to break down extracellularly. Thus, the MIC data for *S. cerevisiae* 9763, *C. albicans* 1-V, and *C. albicans* WD 18-4 show virtually no differences (≤1 dilution) between 5-FC and the 5-FC derivatives. To some extent this consistency is surprising, as we would have expected the wide range of stabilities ($t_{1/2}$ = 0.3–17.6 h) to result in some variability for individual MIC values. The results on the above microorganisms contrast with those obtained with *C. krusei*, in which case the 5-FC conjugates are less active (≤4 dilutions) than the parent drug. If the conjugates were hydrolyzed extracellularly, we would have expected to see consistent results for all the organisms studied, since the same test conditions were used in each case.

The ideal carrier constructed on the basis of the structural specificity for peptide uptake in *C. albicans* WD 18-4 contains a free carboxyl terminus. Thus, the conjugates with 5-FC linked to the carboxyl terminus of the peptide carrier and those containing succinyl peptide ester derivatives were not expected to enter *C. albicans* WD 18-4 via the peptide-transport system. The fact that these compounds and free 5-FC have similar MIC values against the four test organisms suggests that all of the compounds investigated either enter the cell via a route other than the peptide-transport system or that they are hydrolized extracellularly. Our stability studies favor the latter conclusion. The conjugates with free carboxyl termini (compounds **12** and **13**) also show MIC values identical with those for free 5-FC. These derivatives could enter *C. albicans* via its peptide-transport system. The short half-lives determined for compounds **12** and **13** under conditions identical with those used in the biological assay do not allow us to distinguish between entry via the peptide-transport system or extracellular hydrolysis to free 5-FC. We are currently attempting to determine the mode of action of our drug–conjugates by using mutants of yeast lacking either the cytosine permease or the peptide-transport system. Such studies might prove whether 5-FC only enters *C. albicans* in the free form or whether other modes of entry also exist.

The results obtained with the 5-FC derivatives indicate that the type of linkage used to conjugate the peptides may be susceptible to nonspecific hydrolysis and is, therefore, inappropriate for the test conditions employed. We are currently synthesizing model compounds that are expected to be stable to nonenzymatic hydrolysis. These conjugates will allow a more definitive interpretation of the application of peptide as carriers for toxic agents into pathogenic yeasts.

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Nitrosoureidonucleosides

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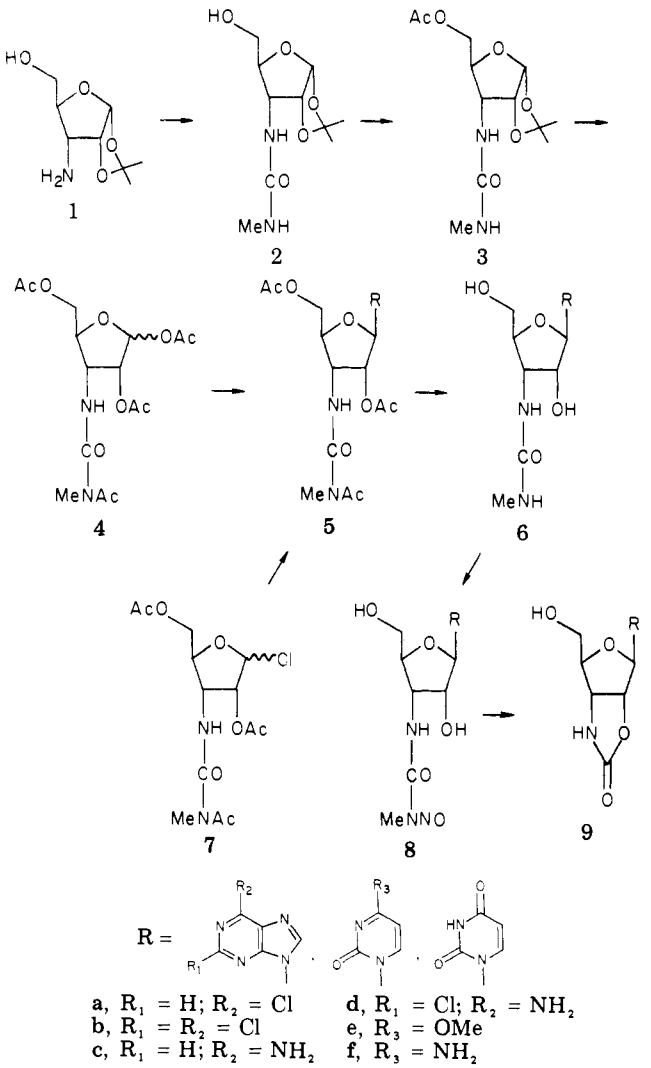
3-Deoxy-1,2-*O*-isopropylidine-3-(3-methylureido)-α-D-ribofuranose (**2**) was converted to 1,2,5-tri-*O*-acetyl-3-deoxy-3-(3-acetyl-3-methylureido)-D-ribofuranose (**4**) and the corresponding glycosyl chloride (**7**). These sugars were converted to the 3-deoxy-3-(3-methylureido)-β-D-ribofuranosyl derivatives of adenine (**6c**), 2-chloroadenine (**6d**), cytosine (**6f**), and uracil (**6g**). Nitrosation of these nucleosides gave the corresponding 3-methyl-3-nitrosoureidonucleosides **8c,d,f,g**. 5′-Amino-5′-deoxyadenosine (**10a**), 5′-amino-5′-deoxyuridine (**10b**), and 5′-amino-5′-deoxycytidine (**10c**) were all converted to the corresponding 5′-(methylureido)-5′-deoxynucleosides **15a–c** by reaction with methyl isocyanate. Nitrosation of these compounds gave the methylnitrosoureidonucleosides **16a–c**. These nitrosoureas, potential active-site-directed irreversible enzyme inhibitors, showed little cytotoxicity or activity against leukemia L1210 in vivo.

Attempts to prepare a purine or pyrimidine containing a chemically reactive function appear to date back to the work of Huber in 1956 on the synthesis of the "adenine mustard" [*N,N*-bis(2-chloroethyl)adenine].[1,2] Later, others were more successful in preparing related structures designed to combine irreversibly with the active site of purine-metabolizing enzymes.[3,4] The activity of the nitrosoureas against experimental animal neoplasms[5] then led to the preparation of purines with side chains containing a nitrosoureido function, one of which showed moderately good activity against leukemia L1210.[6]

These results and our interest in nucleosides caused us to undertake the synthesis of some nucleosides containing a nitrosoureido function in the carbohydrate moiety. Since our interest is in obtaining nucleosides whose activity might be due to their in vivo breakdown to an isocyanate capable of carbamoylating a basic center at the active site[7,8] of a nucleoside- or nucleotide-metabolizing enzyme, we chose the methylnitrosoureido group rather than the 2-chloroethylnitrosoureido group,[9] the activity of which would be more likely to be due to generation of the biologically potent 2-chloroethyldiazo hydroxide.[7,8,11-13] The methylnitrosoureas, on the other hand, show relatively low alkylating potential.[7] We chose to position the latent isocyanate moiety at the 3′ and 5′ positions of the nucleosides. Since coformycin, a nucleoside, is known to be a good inhibitor not only of adenosine deaminase but also of adenylate deaminase,[14] the 3′-deoxy-3′-methylnitrosoureidonucleosides themselves might inhibit nucleotide-metabolizing enzymes or they might be phosphorylated to the corresponding nucleotides, which in turn might be enzyme inhibitors. The 5′-[(methylnitroso)ureido]nucleosides, on the other hand, cannot be phosphorylated but might inhibit nucleotide-metabolizing enzymes by carbamoylating the basic center, usually a guanidinium group, known to be necessary for the binding of the phosphate moiety of nucleotides to the active site of the nucleotide-metabolizing enzymes.[15]

The most reasonable approach to the synthesis of nucleosides containing a 3′-(nitrosoureido) function appeared to be the synthesis of the appropriate ureido sugar for attachment to the desired purine or pyrimidine. Reaction of 3-amino-1,2-*O*-isopropylidine-α-D-ribofuranose (**1**)[16] with methyl isocyanate in ether proceeded in high yield to give 3-deoxy-1,2-*O*-isopropylidene-3-(3-methylureido)-α-D-ribofuranose (**2**) (Scheme I).[17] Acetylation of this compound with acetic anhydride in pyridine took place at the 5-hydroxyl only to give 5-*O*-acetyl-3-deoxy-1,2-*O*-iso-

Scheme I



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