Transport of Antimicrobial Agents Using Peptide Carrier Systems: Anticandidal Activity of *m*-Fluorophenylalanine–Peptide Conjugates[1]

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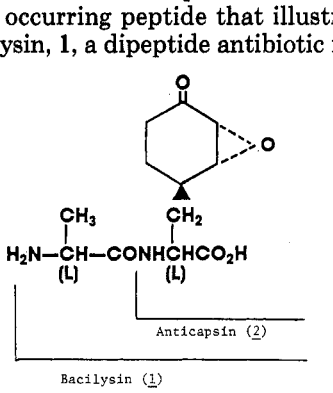
A series of di- and tripeptides containing D- and L-*m*-fluorophenylalanine was prepared and tested in vitro for the ability to inhibit the growth of the yeast *Candida albicans*. The results demonstrate that peptides containing L-*m*-fluorophenylalanine inhibited the growth of *C. albicans* with minimum inhibitory concentrations (MIC's) ranging from 0.5 to 63 μg/mL. The parent L-*m*-fluorophenylalanine and peptides containing D-*m*-fluorophenylalanine were inactive (MIC > 250 μg/mL) in these tests. The results of competitive antagonism studies support peptide transport mediated entry of the inhibitory peptides, followed by release of L-*m*-fluorophenylalanine inside the cell.

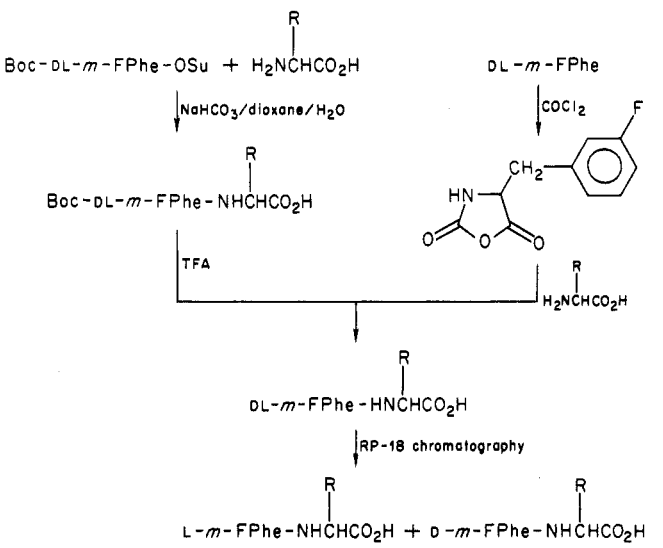
For a substance to be an effective antimicrobial agent it must be able to interfere with an essential function of the microbial cell. Target sites within the cell, such as enzymes, ribosomes, nucleic acids, etc., are often susceptible to inhibitors when tested in cell-free systems, but the intact microbe is often not susceptible to the same agents. This difference in inhibitory activity between intact and cell-free systems is commonly attributed to cell impermeability where elements of the cell membrane restrict access of external molecules from the cell's interior. This permeability barrier is overcome by specific transport systems that allow selected compounds (i.e., nutrients) to enter the cell. Studies using auxotrophic strains of *Candida albicans* have shown that amino acids can be supplied to cells either as the free amino acid or in the form of peptides that contain the required amino acid and that entry of these peptides occurs by an active-transport process mediated by peptide permeases.[2,3] Presumably, once the peptide enters the cell it is hydrolyzed rapidly by intracellular peptidases, resulting in the release of the constituent amino acids. Thus, it is conceivable that amino acid antimetabolites, normally impermeable to cells of *C. albicans*, could be transported into the cells when incorporated into a peptide chain and this concept provides a rational approach for the development of antifungal drugs.[4]

A naturally occurring peptide that illustrates this rationale is bacilysin, **1**, a dipeptide antibiotic isolated from



a culture of *Bacillus subtilis*. Anticapsin (**2**) the "warhead" amino acid of bacilysin and a potent inhibitor of glucosamine synthetase, is poorly active against whole cells but is effectively transported into cells as the peptide.[5] Also, peptides containing *m*-fluorophenylalanine were reported to have activity against the fungal organisms *Paecilomyces varioti* and *Mucor miehei*.[6] Since *m*-fluorophenylalanine is itself a weak inhibitor of fungal growth, it was presumed that the amino acid was carried into the cells as the peptide and hydrolyzed by intracellular peptidases releasing free *m*-fluorophenylalanine. Although the exact mechanism(s) by which *m*-fluorophenylalanine exerts its inhibitory effects has not been established, several possibilities exist, including intracellular conversion of *m*-fluorophenylalanine to fluorocitrate,[7] a potent irreversible inhibitor of aconitase,[8a,b] and/or the synthesis of anomalous malfunctioning proteins containing *m*-fluorophenylalanine.[9]

Scheme I



In this paper, we describe the use of *m*-fluorophenylalanine as the "warhead" component of peptides and the in vitro activity of these peptides against *C. albicans*. Evidence is presented that supports peptide transport mediated entry of these substances into *C. albicans*, followed by the release of *m*-fluorophenylalanine inside the cell.

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(1) Presented at the American Chemical Society 16th Middle Atlantic Regional Meeting, Newark, DE, April 21, 1982.
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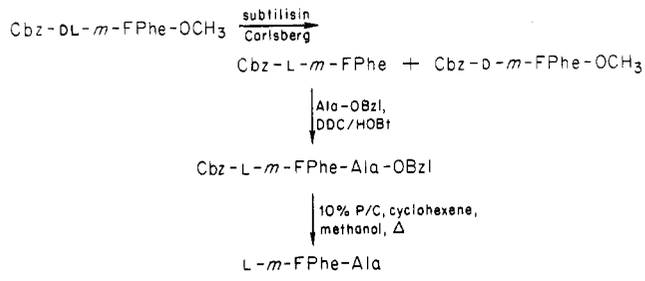
Scheme II



Chemistry. Synthesis of the *m*-fluorophenylalanyl peptides was accomplished by using standard peptide synthetic procedures. Initially, commercial DL-*m*-fluorophenylalanine was used; for example, peptides that contain *m*-fluorophenylalanine as the N-terminal amino acid were prepared by an activated ester displacement between *N*-(*tert*-butoxycarbonyl)-DL-*m*-fluorophenylalanine hydroxysuccinimide ester and the desired free amino acid or dipeptide. Alternatively, the peptides could be prepared from the *N*-carboxyanhydride of *m*-fluorophenylalanine, prepared from *m*-fluorophenylalanine and phosgene, and the amino acid or dipeptide (Scheme I). Peptides that contain the *m*-fluorophenylalanine at the carboxy terminus were prepared by reaction of the *N*-(*tert*-butoxycarbonyl) amino acid or dipeptide hydroxysuccinimide ester and DL-*m*-fluorophenylalanine. The resulting diasteromeric mixtures were separated by reverse-phase chromatography, and stereochemical assignments of the separated diasteromers were initially made based on biological activity. That is, small peptides containing D-amino acids are poorly transported,[2,3] and, therefore, the less active isomer of the diasteromeric pair was assigned D stereochemistry. The stereochemical assignments made, based on biological activity, were later verified by an independent synthesis using L-*m*-fluorophenylalanine, which was prepared according to the procedure of Bosshard and Berger[10] (Scheme II). A correlation of stereochemistry by NMR was not possible due to only modest differences in spectral peak positions between diasteromers (see Experimental Section).

Results and Discussion

The minimum inhibitory concentrations (MIC's) of the *m*-fluorophenylalanyl peptides were determined for two strains of *Candida albicans* by a microtiter broth dilution assay. The results obtained (Table I) permit the following conclusions to be made relative to peptide delivery systems in *C. albicans*: (1) Incorporation of L-*m*-fluorophenylalanine, inactive by itself, into a peptide chain results in compounds with in vitro activity against *C. albicans*, a result consistent with the "portage transport" concept described by Gilvarg.[11] (2) Peptides that contain D-*m*-fluorophenylalanine have greatly reduced activity. This result is consistent with several possibilities, such as the failure of peptides containing D-amino acids to penetrate cells of *C. albicans*,[2-4] resistance to intracellular peptidases, and the possible inactivity of free intracellular D-*m*-fluorophenylalanine. (3) The warhead amino acid may be positioned at either end of the peptide (compare L-Ala-L-Ala-L-*m*-FPhe with L-*m*-FPhe-L-Ala-L-Ala).

If the *m*-fluorophenylalanyl peptides are being transported into fungal cells via peptide permeases, it should be possible to antagonize the inhibitory activity of these peptides by adding nonhibitory peptides that would compete for the permeases. Tables II and III show the results of a series of disk diffusion assays in which cells of *C. albicans* were treated with a disk containing the inhibitory peptide and overlaid with another disk containing a noninhibitory peptide. The results of these experiments show that the inhibitory activity of L-*m*-FPhe-L-Ala is antagonized by the addition of both di- and trialanine (Table II) and that L-*m*-FPhe-L-Ala-L-Ala is antagonized more by trialanine than by dialanine; although our antagonism studies were performed predominantly using alanine peptides, other peptides, such as dimethionine and trimethionine could be anticipated to produce similar results.[3,4] The exact multiplicity of peptide transport permeases in *C. albicans*, that is, either separate permeases for di- and tripeptides or a general peptide permease that accomodates both di- and tripeptides, remains uncertain.[2,3] The results of our studies do not convincingly support either possibility; however, the antagonism of L-*m*-FPhe-L-Ala by both a dipeptide (Ala_2) and a tripeptide (Ala_3) coupled with the extremely weak antagonism of L-*m*-FPhe-L-Ala-L-Ala by dipeptides (i.e., Ala_2 and Gly-Ala) implies either different entry modes for di- and tripeptides or different binding affinities for a common transport system. In order to gain more definitive information on the nature of the peptide transport systems that exist in *C. albicans*, the isolation of selective peptide transport resistant mutants and the measurement of peptide binding constants (i.e., dipeptide vs. tripeptide) will be useful. Efforts directed toward both of these aspects are in progress, and the results of these studies will be reported when completed.

Table I. Activity of *m*-Fluorophenylalanine Peptides against *C. albicans*

compound	MIC, μG/mL, against *C. albicans* strains B311[a]	759[a]
L-*m*-FPhe	>250	>250
L-*m*-FPhe-L-Ala	2	4
D-*m*-FPhe-L-Ala	>200	>200
L-*m*-FPhe-L-Ala-L-Ala	0.5	2
D-*m*-FPhe-L-Ala-L-Ala	>250	>250
L-*m*-FPhe-L-Met-L-Met	8	63
D-*m*-FPhe-L-Met-L-Met	>250	>250
L-Ala-L-Ala-L-*m*-FPhe	2	63

[a] Inoculum size 10^4 cfu/mL. Incubation: 37 °C, 48 h.

The surprisingly potent antagonism of the inhibitory tripeptide L-*m*-FPhe-L-Ala-L-Ala by the dipeptide Gly-Phe was speculated to be a result of increased intracellular concentrations of phenylalanine that could compete with *m*-fluorophenylalanine, presumably at a cytoplasmic enzyme site, and not a result of competition for the oligopeptide transport permease. In order to test this speculation, 28 amino acids were codisked with L-*m*-FPhe-L-Ala-L-Ala. The only amino acid found to antagonize the activity of the inhibitory peptide was phenylalanine (Table IV shows an abbreviated list of amino acids tested). This result implies that the active agent within the cells is L-*m*-fluorophenylalanine and not a toxic peptide, since antagonism of peptides by amino acids (i.e., phenylalanine) would not be expected.

Our results demonstrate that L-*m*-fluorophenylalanine can be transported into *C. albicans* by peptide carriers. Presumably, after being transported into the cells, the peptides are hydrolyzed by intracellular peptidases, resulting in the release of the toxic warhead L-*m*-fluorophenylalanine. Furthermore, this study supports the hypothesis that the attachment of an antimetabolite to a

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Table II. Antagonism of L-m-Fluorophenylalanyl-L-alanine Activity by Dipeptides and Tripeptides

concn of L-m-FPhe-L-Ala, μg/disc	zone size, mm, against *C. albicans*[a]				
	control	Ala-Ala (200 μg/disk)	Ala-Ala-Ala (200 μg/disk)	Gly-Ala (200 μg/disk)	Gly-Phe (200 μg/disk)
25.0	25	0	0	25–31	0
12.5	16	0	0	0	0
6.0	13	0	0	0	0
3.0	9	0	0	0	0

[a] Peptides were codisked on L-m-FPhe-L-Ala disks.

Table III. Antagonism of L-m-Fluorophenylalanyl-L-alanyl-L-alanine by Dipeptides and Tripeptides

concn of L-m-FPhe-L-Ala-L-Ala, μg/disc	zone size, mm, against *C. albicans*[a]				
	control	Ala-Ala (200 μg/disk)	Ala-Ala-Ala (200 μg/disk)	Gly-Ala (200 μg/disk)	Gly-Phe (200 μg/disk)
25.0	24	24 (hazy)	0	25 (hazy)	0
12.5	20	18 (hazy)	0	18 (hazy)	0
6.0	17	16 (hazy)	0	0	0
3.0	12	0	0	0	0

[a] Peptides were codisked on L-m-FPhe-L-Ala disks.

Table IV. Effect of Amino Acids on Activity of L-m-FPhe-L-Ala-L-Ala

amino acids[a] combined with L-m-FPhe-L-Ala-L-Ala[b]	zone sizes, mm, against *C. albicans* B-311
L-m-FPhe-L-Ala-L-Ala:	24
+ aspartic acid	26
+ histidine	25
+ isoleucine	27
+ phenylalanine	0
+ tryptophan	24
+ proline	27

[a] 250 μg/disk of amino acid codisked with 12.5 μg/disk of L-m-FPhe-L-Ala-L-Ala. [b] 12.5 μg/disk of L-m-FPhe-L-Ala-L-Ala.

carrier peptide provides a viable mechanism for the introduction of otherwise impermeable molecules into fungal cells and provides a rational approach to the discovery of novel antifungal agents.[4,11]

Experimental Section

Elemental analyses were performed in the Chemical Technologies Department of Smith Kline & French Laboratories. IR spectra were obtained with a Perkin-Elmer 137 spectrophotometer. NMR spectra were obtained on either a Varian T-60 or a Varian FT-80A spectrometer [$(CH_3)_4Si$ or SDSS]. Although IR and NMR spectral data are reported only where considered significant, spectra were obtained for all compounds described and were evaluated as consistent with assigned structures. Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter. Reactions were monitored by TLC using the following systems: (I) Whatman KIF silica gel, methylene chloride/methanol (19:1 v/v); (II) Whatman KC18F reverse phase, methanol/5% aqueous NaCl (1:1, v/v); (III) Analtech MN 300 cellulose, butanol/acetic acid/water (4:1:4, v/v). Preparative chromatography was achieved by using medium-pressure chromatography (MPLC) with a 25 × 250 mm Altex column packed with EM silica gel (40–60 μm, 5–20 psi) for the blocked peptides and a 25 × 500 mm Altex column packed with EM Lichroprep RP-18 (25–40 μm, 50–90 psi) for the deblocked peptides.

Boc-DL-m-FPhe. To a mixture of 2.0 g (10.9 mmol) of DL-m-FPhe (Sigma Chemical Co.), 1.64 g (16.4 mmol) of Et_3N, and 7 mL of water was added 3.96 g (12.0 mmol) of Boc-ON (Aldrich Chemical Co.) dissolved in 7 mL of dioxane. The resulting mixture was stirred for 2.5 h, treated with 20 mL of water, washed twice with 30 mL of EtOAc, and acidified with 10% citric acid to pH 3.5. The resulting aqueous mixture was extracted twice with 30 mL of EtOAc, and the combined organic extracts were dried ($MgSO_4$) and concentrated to give 2.11 g (68%) of Boc-DL-m-Phe as a white solid, which was recrystallized from hexane/ethyl ether (3:1, v/v). TLC (system I) indicated pure product (R_f 0.47): NMR [$CDCl_3$, $(CH_3)_4Si$] δ 7.6–6.75 (m, 4 H), 5.4–4.9 (br, 1 H), 4.8–4.2 (br m, 1 H), 3.2–3.0 (m, 2 H), 1.5 (s, 9 H).

Boc-DL-m-FPhe-OSu. To 1.85 g (6.54 mmol) of Boc-DL-m-FPhe and 0.75 g (6.54 mmol) of *N*-hydroxysuccinimide was added 1.48 g (7.2 mmol) of DCC dissolved in 70 mL of THF. The reaction mixture stirred overnight, several drops of acetic acid were added, and the mixture was stirred for an additional 0.5 h and filtered. Concentration gave 2.7 g of crude product, which was dissolved in warm toluene and filtered. Pure Boc-DL-m-FPhe-OSu crystallized on cooling to give 1.49 g (60%) of a white solid: NMR [$(CD_3)_2CO$, $(CH_3)_4Si$] δ 7.6–6.9 (m, 4 H), 4.9 (br m, 2 H), 3.8 (s, 4 H), 3.3–3.1 (m, 2 H), 1.4 (s, 9 H).

Boc-DL-m-FPhe-L-Ala-L-Ala. To a 0.317-g (1.98 mmol) portion of L-Ala-L-Ala (Sigma Chemical Co.) and 0.33 g (3.96 mmol) of $NaHCO_3$ in 11 mL of water was added 0.75 g (1.98 mmol) of Boc-DL-m-FPhe-OSu dissolved in 12 mL of dioxane. TLC (solvent system I) indicated that the reaction was complete after 2 h, after which the solvent was evaporated, the residue was combined with 15 mL water and 15 mL EtOAc, and the pH was adjusted from 7.5 to 3.2 with 10% citric acid. The resulting aqueous solution was extracted twice with EtOAc (15 mL), and the combined organic extracts were washed with 10% citric acid (2 × 15 mL) and brine (20 mL) and dried ($MgSO_4$). Concentration gave 0.69 g (82%) of product containing a minor impurity (TLC, system I), which was removed by stirring briefly in hot CH_2Cl_2; NMR [$(CD_3)_2CO$, $(CH_3)_4Si$] δ 6.7–5.5 (m, 4 H), 4.7–4.1 (m, 3 H), 3.2–2.8 (m, 2 H), 1.5–1.1 (m, 15 H).

DL-m-FPhe-L-Ala-L-Ala. To 0.49 g (1.15 mmol) of Boc-DL-m-FPhe-L-Ala-L-Ala cooled to 10 °C was added 5 mL of TFA and 0.5 mL of anisole. After the mixture was stirred for 0.5 h, the cooling bath was removed, and the reaction mixture was stirred at room temperature for an additional 1.5 h. TLC (system I) showed complete disappearance of starting material. The reaction mixture was then poured into 125 mL of Et_2O, and the precipitated solid was filtered, dissolved in H_2O, and stirred with IR-45 (OH^-) until a pH of 5.9 was obtained. The resulting mixture was filtered to remove the resin, and the aqueous solution was lyophilized to give 0.15 g of a white solid: IR (KBr) 3600–1900, 1640 cm^{-1}; NMR (D_2O, SDSS) δ 7.5–6.9 (m, 4 H), 4.5–4.0 (m, 3 H), 3.4–3.0 (m, 2 H), 1.6–1.0 (m, 6 H). TLC (solvent system II) indicated a separable mixture of diasteromers (R_fs 0.82 and 0.74).

Separation of DL-m-FPhe-L-Ala-L-Ala Diasteromers. A 100-mg portion of the above diasteromeric mixture was eluted from a preparative reverse-phase column (Lichroprep RP-18) using MPLC conditions and eluting with a solvent sequence of 0, 10, 20, and 30% methanol/water. The desired isomer eluted in the 20 and 30% methanol/water mixtures.

The first isomer eluted weighed 52 mg after lyophilization: $[\alpha]^{25}_D$ −28.2° (*c* 1, 1 N HCl); NMR (D_2O, SDSS) δ 7.5–6.9 (m, 4 H), 4.5–4.0 (m, 3), 3.2 (br m, 2 H), 1.5 (d, 6 H, J = 8 Hz); IR (KBr) 3600–1900, 1640 cm^{-1}. Anal. ($C_{15}H_{20}FN_3O_4 \cdot {}^2/_3H_2O$) C, H, N.

The second diasteromer weighed 32 mg after lyophilization: $[\alpha]^{25}_D$ −108.2° (*c* 1, H_2O); NMR (D_2O, SDSS) δ 7.5–6.9 (m, 4 H), 4.5–4.0 (m, 3 H), 3.4–3.0 (m, 2 H), 1.6 (d, 3 H, *J* = 8 Hz), 1.4 (d, 3 H, *J* = 8 Hz); IR (KBr) 3600–1900, 1640 cm^{-1}. Anal. ($C_{15}H_{20}FN_3O_4 \cdot {}^7/_8H_2O$) C, H, N.

Cbz-L-*m*-FPhe-L-Ala-L-Ala-OBzl. To 0.287 g (1.0 mmol) of L-Ala-L-Ala-OBzl·HCl and 0.115 g (1.0 mmol) of *N*-ethylmorpholine dissolved in 5 mL of DMF and cooled to 5–10 °C were added 0.317 g (1.0 mmol) of Cbz-L-*m*-FPhe,[9] 0.135 g (1.0 mmol) of hydroxybenzotriazole, and 0.227 g (1.1 mmol) of DCC. The resulting mixture was stirred under cooling for 1 h and at 27 °C for 2 h and then filtered, and the filtrate was concentrated, dissolved in 50 mL of EtOAc, washed with 5% $NaHCO_3$ (2 × 20 mL), 10% citric acid (2 × 20 mL), 5% $NaHCO_3$ (2 × 20 mL), and brine (20 mL), and dried ($MgSO_4$). Concentration gave 0.5 g (92%) of slightly impure product (TLC, system I), which was chromatographed on a silica gel column (MPLC), eluting with 2% CH_3OH in CH_2Cl_2 to give 0.443 g (69%) of purified solid: NMR [$CDCl_3$, $(CH_3)_4Si$] δ 7.6–6.7 (m, 16 H, NH and aromatics), 6.0 (d, 1 H, NH), 5.17 (s, 2 H), 5.07 (s, 2 H), 4.9–4.2 (m, 3 H), 3.0 (d, 2 H), 1.5–1.1 (m, 6 H).

L-*m*-FPhe-L-Ala-L-Ala. A 0.22-g (0.40 mmol) portion of Cbz-L-*m*-FPhe-L-Ala-L-Ala-OBzl was combined with 0.20 mL (0.16 g, 2.0 mL) of cyclohexene and 0.20 g of 10% Pd/C in 10 mL of CH_3OH. The resulting mixture was refluxed for 3 h, after which time the starting material was completely reacted (TLC, system I). The hot reaction mixture was filtered and concentrated to give 0.107 g (82%) of a solid whose TLC (solvent system II) and NMR spectrum matched exactly with that of the first diasteromer eluted on chromatography of the DL-*m*-FPhe-L-Ala-L-Ala mixture. There was no trace of the other diasteromer (D-*m*-FPhe-L-Ala-L-Ala), as evidenced by TLC (systems II and III). A portion of this material was chromatographed (MPLC) on an RP-18 column and lyophilized to give a white solid: $[\alpha]^{25}_D$ −28.2° (*c* 1, 1 N HCl). Anal. ($C_{15}H_{20}FN_3O_4 \cdot {}^3/_4H_2O$) C, H, N.

DL-*m*-FPhe-L-Met-L-Met. Boc-DL-*m*-FPhe-L-Met-L-Met was prepared by the activated ester procedure as described for Boc-DL-*m*-FPhe-L-Ala-L-Ala to give the desired product in 95% crude yield: NMR [$CDCl_3$, $(CH_3)_4Si$] δ 7.7–6.7 (m, 7 H), 4.8–4.2 (m, 3 H), 3.1–2.9 (m, 2 H), 2.7–1.6 (m, 14 H), 1.3 (s, 9 H).

The blocking groups were removed by use of TFA–anisole, followed by IR-45 (OH) treatment as previously described, to give the desired product in 27% yield. As was demonstrated for the alanine tripeptide, the DL-*m*-FPhe-L-Met-L-Met diasteromers were separable on reverse-phase TLC (system II): NMR [TFA-*d*, $(CH_3)_4Si$] δ 7.8–6.8 (m, 4 H), 5.2–4.5 (m, 3 H), 3.3 (br d, 2 H), 3.0–1.8 (m, 14 H).

Separation of DL-*m*-FPhe-L-Met-L-Met Diasteromers. A 0.187-g portion of the DL mixture was chromatographed (MPLC) on a C-18 reverse-phase column using a stepwise gradient of 0, 10, 20, 30, and 40% CH_3OH in water. The desired diasteromers were eluted in 40% CH_3OH. Stereochemical assignments were based on the analogy with the chromatographic and biological properties of the *m*-FPhe-(L-Ala)$_n$ peptides.

The first diasteromer to elute was L-*m*-FPhe-L-Met-L-Met (51 mg): NMR (D_2O, SDSS) δ 7.6–6.6 (m, 4 H), 4.5–3.7 (m, 3 H), 3.2 (br d, 2 H), 2.7–2.3 (m, 4 H), 2.2–1.8 (m, 10 H); IR (KBr) 3600–1900, 1660, 1580 cm^{-1}. Anal. ($C_{19}H_{28}FN_3O_4S_2 \cdot H_2O$) C, H, N.

The second diasteromer to elute was the D-*m*-FPhe-L-Met-L-Met (38 mg): NMR (D_2O, SDSS) δ 7.8–6.8 (m, 4 H), 4.5–3.6 (m, 3 H), 3.2–2.8 (m, 2 H), 2.7–2.3 (m, 4 H), 2.2–1.8 (m, 10 H); IR (KBr) 3600–1900, 1640, 1580. Anal. ($C_{19}H_{28}FN_3O_4S_2 \cdot 1{}^1/_8H_2O$) C, H, N.

Cbz-L-*m*-FPhe-L-Ala-OBzl. A 0.216-g (1.0 mmol) sample of L-Ala-OBzl (Sigma Chemical Co.) and 0.317 g (1.0 mmol) of Cbz-L-*m*-FPhe (**9**) were coupled using DCC by the procedure previously described in the preparation of Cbz-L-*m*-FPhe-L-Ala-L-Ala-OBzl to give 91% of crude product, which was purified by chromatography on silica gel (MPLC) to give 0.31 g (65%) of a foam, which was a single spot (R_f 0.50) on TLC (system I): NMR [$CDCl_3$, $(CH_3)_4Si$] δ 7.5–6.6 (m, 15 H), 5.6 (br d, 1 H), 5.2 (s, 2 H), 5.1 (s, 2 H), 4.5 (m, 2 H), 3.0 (d, 2 H), 1.3 (d, 3 H).

L-*m*-FPhe-L-Ala. A 0.312-g (0.65 mmol) sample of Cbz-L-*m*-FPhe-L-Ala-OBzl was deblocked as previously described to give 0.193 g (75%) of the desired dipeptide. TLC (systems II and III) indicated a single product: NMR (D_2O, SDSS) δ 7.7–6.8 (m, 4 H), 4.5–4.0 (m, 2 H), 3.5–3.2 (m, 2 H), 1.4 (d, 3 H); IR (KBr) 3600–2200, 1650 cm^{-1}.

An analytical sample was prepared by chromatography on a RP-18 column to give, after lyophilization, a white solid: $[\alpha]^{25}_D$ 16.4° (*c* 1, H_2O). Anal. ($C_{12}H_{16}FN_2O_3 \cdot {}^1/_2H_2O$) C, H, N.

DL-*m*-FPhe-*N*-carboxyanhydride. A 10.0-g (0.55 mol) portion of DL-*m*-fluorophenylalanine in 200 mL of dry THF was refluxed for 2 h while phosgene gas was passed through the solution. The reaction mixture was cooled and concentrated. Recrystallization of the resulting residue from toluene gave 8.84 g (77%) of a white solid: mp 116 °C; IR (CH_2Cl_2) 3440, 1870, 1800 cm^{-1}.

DL-*m*-FPhe-L-Ala. A mixture consisting of 3.12 g (35.1 mmol) of L-alanine, 3.51 g (33.1 mmol) Na_2CO_3, 35 mL of 1 N NaOH, 140 mL of water, and 140 mL of acetonitrile was cooled to −10 °C, and 8.8 g (42.1 mmol) of DL-*m*-FPhe-*N*-carboxyanhydride, prepared as described above, was added. The resulting mixture was stirred at −10 °C for 3 h, after which the acetonitrile was separated from the aqueous phase. The aqueous phase was adjusted to pH 6 with H_2SO_4 and partially desalted by addition of ethanol. The salts were filtered, the filtrate was concentrated, and the residue was passed down a Sephadex G-10 column (2.6 × 100 cm) to give 3.75 g (42%) of semipure product. Further purification and separation of diasteromers were accomplished by preparative reverse-phase (C-18) chromatography as described above to give L-*m*-FPhe-L-Ala, which was identical with the authentic sample prepared from L-*m*-FPhe as described above and D-*m*-FPhe-L-Ala: $[\alpha]^{25}_D$ −93.8° (*c* 1, H_2O); NMR (D_2O, SDSS) δ 7.6–7.0 (m, 4 H), 4.4–3.8 (m, 2 H), 3.3 (dd, 2 H), 1.2 (d, 3 H); IR (KBr) 3600–2200, 1650 cm^{-1}. Anal. ($C_{12}H_{16}FN_2O_3 \cdot H_2O$) C, H, N.

L-Ala-L-Ala-DL-*m*-FPhe. DL-*m*-FPhe was coupled to the activated ester Boc-L-Ala-L-Ala-OSu[12] by the activated ester procedure as previously described to give Boc-L-Ala-L-Ala-DL-*m*-FPhe in 85% crude yield. Purification by MPLC chromatography (CH_2Cl_2, 5% CH_3OH, silica gel) gave the desired product [NMR [$CDCl_3$, $(CH_3)_4Si$] δ 8.0 (m, 2 H), 7.5–6.7 (m, 4 H), 5.5 (m, 1 H), 4.8–3.8 (m, 3 H), 3.1 (m, 2 H), 1.4 (s, 9 H), 1.3 (d, 6 H)], which was deblocked as described previously to give L-Ala-L-Ala-DL-*m*-FPhe as a mixture of diasteromers (HPLC, Lichrosorb C-18, 10 mm, 250 × 4 mm, 25% CH_3OH in H_2O). Preparative reverse-phase chromatography (C-18) produced L-Ala-L-Ala-L-*m*-FPhe (biologically active), which contained approximately 5% of the D diasteromer as assayed by HPLC: NMR (D_2O, SDSS) δ 7.4–6.7 (m, 4 H), 4.9 (dd, 1 H), 4.6 (q, 1 H), 4.2 (q, 1 H), 3.2 (dd, 2 H), 1.5 (d, 3 H), 1.3 (d, 3 H); IR (KBr) 3600–2300, 1635 cm^{-1}. Anal. ($C_{15}H_2OFN_3O_4 \cdot {}^1/_2H_2O$) C, H, N.

Biological Testing. (A) Minimum Inhibitory Concentration (MIC). *Candida albicans* strains were from the SK&F collection. The MIC's of the compounds were determined by twofold broth dilution tests in yeast carbon base medium (YCB, Difco) containing 200 μg/mL of sodium glutamate. The compounds were diluted from 250 to 0.25 μg/mL, and the test was inoculated with a suspension of *C. albicans* grown in YCB–sodium glutamate for 15 h at 37 °C. The final inoculum sizes in the tests were approximately 10^4 cfu/mL. The tests were incubated at 37 °C for 24 and 48 h and observed for inhibition of growth.

(B) Disk Diffusion Assay. Seeded plates of *C. albicans* for the disk diffusion assay were prepared as follows: *C. albicans* slant culture was grown in trypticase soy broth (BBL) for 7 h at 37 °C on a New Brunswick rotary shaker set at 250 rpm. One liter of yeast carbon base with lysine (200 μg/mL) agar medium was inoculated with 1 mL of the above inoculum. The seeded agar (15 mL) was poured into 150-mm Petri dishes. The peptide

(12) In the final stages of the preparation of this manuscript, a report appeared by W. Meyer-Glauner, E. Bernard, D. Armstrong, and B. Merrifield *Zentralbl. Bakteriol. Parasitenkd., Infektionskr. Hyg., Abt. 1, Orig., Reike A*, **252**, 274–278 (1982)] which describes the lethal effects of *m*-fluorophenylalanine peptides against *C. albicans* but does not explicitly demonstrate peptide transport as the means of entry of these peptides.

(13) R. P. Sharma, M. G. Gore, and M. Akhtar, *J. Chem. Soc., Chem. Commun.*, 875–877 (1979).

drugs were dissolved in water and absorbed onto 6.35-mm Schleicher & Schuell disks and placed on the seeded plates. For antagonism studies, various amino acids and di- and tripeptides were codisked with the peptide drugs.

Acknowledgment. We thank Drs. George Dunn and David Berges for helpful suggestions during the preparation of this paper and Dr. Charles Gilvarg for his excellent advice during the progress of this research.

Registry No. Boc-DL-*m*-FPhe, 87184-23-4; DL-*m*-FPhe, 2629-54-1; Boc-DL-*m*-FPhe-OSu, 87184-24-5; Boc-DL-*m*-FPhe-L-Ala-L-Ala, 87184-25-6; L-Ala-L-Ala, 1948-31-8; DL-*m*-FPhe-L-Ala-L-Ala, 87184-26-7; L-*m*-FPhe-L-Ala-L-Ala, 87184-16-5; D-*m*-FPhe-L-Ala-L-Ala, 87184-17-6; Cbz-L-*m*-FPhe-L-Ala-L-Ala-OBzl, 87184-27-8; L-Ala-L-Ala-OBzl·HCl, 69871-83-6; Cbz-L-*m*-FPhe, 49759-64-0; DL-*m*-FPhe-L-Met-L-Met, 87184-29-0; Boc-DL-*m*-FPhe-L-Met-L-Met, 87184-28-9; L-*m*-FPhe-L-Met-L-Met, 87184-18-7; D-*m*-FPhe-L-Met-L-Met, 87184-19-8; Cbz-L-*m*-FPhe-L-Ala-OBzl, 87184-30-3; L-Ala-OBzl, 17831-01-5; L-*m*-FPhe-L-Ala, 87184-20-1; DL-*m*-FPhe-*N*-carboxyanhydride, 87184-31-4; DL-*m*-FPhe-L-Ala, 87184-32-5; L-Ala, 56-41-7; D-*m*-FPhe-L-Ala, 87184-21-2; L-Ala-L-Ala-DL-*m*-FPhe, 87184-34-7; Boc-L-Ala-L-Ala-OSu, 67818-94-4; Boc-L-Ala-L-Ala-DL-*m*-FPhe, 87184-33-6; L-Ala-L-Ala-L-*m*-FPhe, 87184-22-3; L-Ala-LAla-D-*m*-FPhe, 87184-35-8; L-*m*-FPhe, 19883-77-3.

Synthesis of a Biologically Active Fluorescent Muramyl Dipeptide Congener

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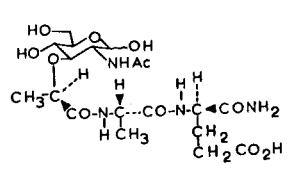
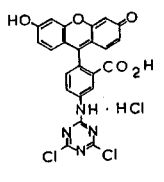
A fluorescent-labeled muramyl dipeptide (MDP) has been prepared to probe immunoadjuvant cellular interactions. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (1) was synthesized in improved yield and reacted with 2-(fluoresceinylamino)-4,6-dichloro-*s*-triazine (DTAF, 2) to give the fluorescent adduct DTAF–MDP (3), attached through the 6-position of the sugar moiety. Adjuvant activity was assessed by using two different in vitro assays, macrophage spreading, and inhibition of macrophage migration. Both assays indicated that the apparent adjuvant activity of 3 is comparable to that of 1.

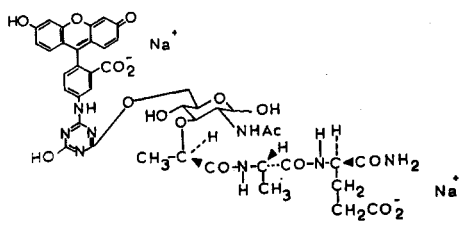
Studies into the adjuvant active fractions of lysozyme digested mycobacteria cell walls led Ellouz et al.[1,2] to propose that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1)



1



2



3

(muramyl dipeptide, MDP) is the minimum structure required which expresses the full spectrum of adjuvant activity when substituted for mycobacteria in Freund's complete adjuvant (FCA). MDP is known to have many effects on both the humoral- and cell-mediated immune systems, and MDP has been implicated in the stimulation of macrophages.[3-7]

The total synthesis[2,8-11] and structure–activity relationship (SAR) studies[11-15] have been performed on MDP. SAR studies revealed several interesting aspects that are relevant to the studies reported here. The C-6 hydroxyl moiety on the muramyl portion of the molecule can be acylated without changing the adjuvant activity.[13] The C-6 hydroxyl group can be converted to an amino function without affecting the activity.[14] The amino acid L-alanine can be replaced by various L amino acids without a significant change in activity.[15] The γ-glutamyl carboxyl group can be converted to an ester or amide or coupled to other amino acids without a change in the biological activity.[11]

Radiolabeled derivatives of MDP have been prepared[16,17] to study the distribution of MDP in the body and to help determine the mechanisms of action for the different adjuvant and immunogenic properties. MDP was found to be distributed throughout the body without substantial

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