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Synthesis of Decapeptide of L-Aspartic Acid and Benzyl-L-Aspartic Acid by Solid Phase Peptide Synthesis

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Polyene macrolide amphotericin B (AmB) is the drug of choice for the treatment of disseminated fungal infections. However, because of its pronounced side effects, the drug has limited applicability. There are few interesting reports, which state that co-administration of the drug with homo-peptide of polyaspartic acid reduces the side effects of the drug. In our present study, an approach has been made to systematically synthesize low molecular weight hetero-peptides consisting of L-aspartic acid and its derivative. It was hypothesized that such hetero-peptides will reduce the toxic side effects of the drug by facile hydrophobic binding between the polymer and the drug. We have employed the strategy of solid phase peptide synthesis (SPPS) to synthesize low molecular weight hetero-peptides by using L-aspartic acid and benzyl-L-aspartic acid to induce the hydrophobic binding between the peptide and the drug. In future, the proposed methodology can be employed to tailor other polypeptides substituted with benzyl groups to reduce the nephrotoxicity of AmB.

Key words: Amphotericin B, Side effects, Decapeptides, L-Aspartic acid, Solid phase peptide synthesis

INTRODUCTION

As a part of ongoing research in our laboratory, we have focused our attention on reducing the side effects of AmB by administration of the drug with hetero-peptides comprising of L-aspartic acid and benzyl-L-aspartic acid. Invasive systemic fungal infections are important causes of morbidity and mortality among immunodeficient patients who are under aggressive chemotherapy, organ transplantation, and HIV infection. Polyene macrolide (AmB) is the drug of choice for the treatment of most systemic fungal infections such as invasive pulmonary aspergillosis and disseminated mycosis (Warnock, 1991; Brime *et al.*, 2004). However, usefulness of the drug is limited due to its pronounced side effects like flu-like symptoms and nephrotoxicity. Up to 80% of patients receiving AmB treatment developed some degree of renal impairment (Bodey *et al.*, 1994; Meyer *et al.*, 1973; Branch, 1988), which may warrant a dose reduction or even cessation of treatment (Bodey *et al.*, 1994; Meyer *et al.*, 1973; Branch, 1988).

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During the last decade, new AmB preparations which are formulation of lipids such as liposome and lipid complex and possess reduced toxicity profile have been developed and marketed (Berman, 1997; Mullen *et al.*, 1997; Townsend *et al.*, 2001; Saxena and Ghosh, 2004; Wiebe and DeGregorio, 1988). The chemical modifications of AmB molecule have also been investigated to reduce its toxicity and to improve solubility (Brajtburg *et al.*, 1990; Borowski, 2004).

There was an interesting report on renal protection upon co-administration of the drug with homo-peptide of polyaspartic acid (Kishore *et al.*, 1990a, 1990b). From a similar font of work, it was speculated that the reduced renal side effect may be attributed to electrophysiological alterations in the drug due to presence of polyaspartic acid (Kaloyanides, 1994). However, there has been no other further research on developing conjugates of drug apart from the homo-peptide of polyaspartic acid with molecular weight of 9,000-15,000. On the basis of previous findings, we have undertaken a project to investigate the effect of low molecular weight hetero-peptides consisting of L-aspartic acid and benzyl-L-aspartic acid on the side effect of AmB.

Our present study involves the synthesis of decapeptide

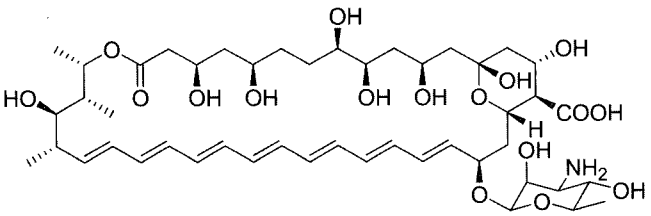


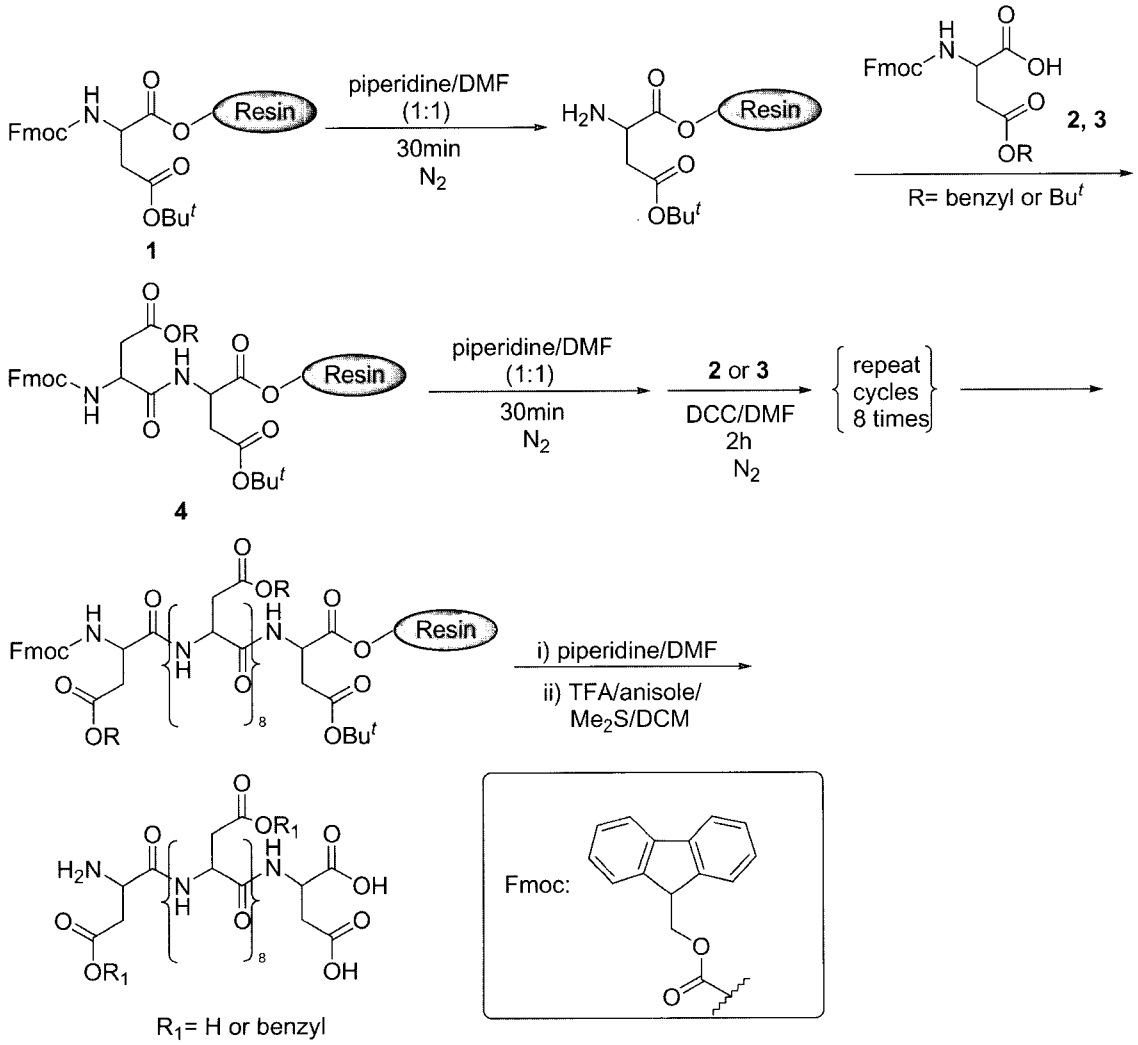
Fig. 1. Chemical structure of amphotericin B

of L-aspartic acid and its derivative, benzyl-L-aspartic acid. AmB is an amphiphilic molecule, which possesses two distinct moieties, i.e., hydrophilic and hydrophobic parts as shown in Fig. 1. Therefore, we have hypothesized that hetero-peptides of aspartic acid and its hydrophobic derivative which mimicks AmB's amphiphilicity and possesses similar molecular weight may be an efficient systems to achieve our goal. The number of amino acid residues in these target peptides was ten and the molecular weight ranged between 1,300 and 1,600. Herein, we report the synthesis of the hetero-peptides of L-aspartic acid and benzyl-L-aspartic acid, which mimic the molecular structure of AmB by following SPPS methodology (Merrifield, 1963). Till date, there have been no reports on the synthesis of the hetero-peptide of the aspartic acid and its derivatives by SPPS methodology.

MATERIALS AND METHODS

Chemicals

Resin (*N*-Fmoc-4-*t*-butyl-L-aspartic acid SASRIN resin ester (**1**), Lot number 095H0704, 0.50 mmol/g), protected benzyl-L-aspartic acid (Fmoc-L-aspartic acid-4-benzyl ester (**2**)), piperidine, and 9-Fluorenyl methyl chloroformate were purchased from Sigma and were used as obtained. Protected L-aspartic acid (*N*-Fmoc-L-aspartic acid-4-*t*-butyl ester (**3**)) was purchased from Fluka and was used without any further purification. Dicyclohexylcarbodiimide (DCC), trifluoroacetic acid (TFA), and methyl disulfide



Scheme 1. Synthesis of decapeptide consisting of L-aspartic acid and benzyl-L-aspartic acid

were obtained from Aldrich. Other reagents and solvents such as dimethylformamide (DMF), methanol, ethanol, dichloromethane (DCM), and anisole (Junsei) were used as obtained from commercial sources.

Synthetic procedure of hetero-peptide

Decapeptide synthesis of L-aspartic acid and benzyl-L-aspartic acid was performed manually on a sintered glass crucible, which was equipped with a nitrogen cylinder and a vacuum pump. Resin (**1**) loaded with 0.50 mmol amino acid per gram was used as a solid support, and Fmoc-protected benzyl-L-aspartic acid (**2**) and *t*-butyl-L-aspartic acid (**3**) were coupled in the presence of DCC according to standard Fmoc-SPPS methodology (Carpino and Han, 1972; Sheppard, 1986; Miranda and Alewood, 1999). Detailed synthetic procedures are illustrated in Scheme 1 and described below.

Step 1:
Suspension of resin (0.16 mmol) in DMF (20 mL) was taken in a sintered glass crucible and was purged with nitrogen gas for 5 min. DMF was filtered off by suction filtration and the process was repeated again by purging nitrogen gas in DMF suspension for 5 more min.

Step 2:
Deprotection of terminal Fmoc group attached to the resin was carried out by treating the resin with piperidine-DMF (1:1 v/v, 20 mL) twice for 5 and 25 min, successively. After removing piperidine and DMF *via* suction filtration, the resin was washed with 20 mL of DMF three times for 3 min.

Step 3:
The deprotected resin was then allowed to couple with five-fold excess amount of protected-benzyl-L-aspartic acid (0.80 mmol, **2**) or protected-L-aspartic acid (0.80 mmol, **3**) in the presence of four-fold excess amount of DCC (0.64 mmol) in DMF (20 mL) under nitrogen gas purging for 2 h. After removing DMF by suction filtration, the solid mixture was thoroughly washed in the order of DMF (2×20 mL), MeOH (2×20 mL), and DMF (20 mL) for 2 min in each step to form resin bound dipeptide (**4**). Steps 2 and 3 were repeated until resin bound protected decapeptide of L-aspartic acid and benzyl-L-aspartic acid was obtained.

Step 4:
The terminal *N*-Fmoc group of resin-bound decapeptide was deprotected by treating the resin with piperidine-DMF (1:1 v/v, 20 mL) according to the same procedure as described in Step 2.

Step 5:
After replacing the collecting flask with a fresh flask, the *t*-butyl groups of resin-bound decapeptide and resin were cleaved by using the mixture of reagents, i.e., TFA, anisole, dimethyl sulfide, and DCM in a volumetric ratio of 30:5:5:60. The mixture of the reagents (25 mL) was added to the sintered glass crucible and stirred by nitrogen gas purging for 45 min. Subsequently, the mixture was filtered off *via* suction and the filtrate was collected in the collecting flask. The sintered glass crucible was washed successively with DCM (2×20 mL) and EtOH (2×20 mL) and the resultant liquid was collected in the flask. The product and combined solvents were concentrated under reduced pressure. Remaining anisole was then driven off under high vacuum by warming the flask in a stream of hot air. The decapeptide of L-aspartic acid and benzyl-L-aspartic acid was dissolved in MeOH followed by slow addition of diethyl ether to induce precipitation of the product.

RESULT AND DISCUSSION

A stepwise SPPS methodology was used for the preparation of decapeptides comprising of L-aspartic acid and benzyl-L-aspartic acid. The design of the synthesis and synthetic approaches were performed on the basis of following considerations. The amino group of L-aspartic acid and benzyl-L-aspartic acid should be protected by base-labile Fmoc group. L-Aspartic acid contains β-carboxylic side chain, which should be protected by base-stable and acid-labile protecting group, for the survival during deprotection of base-labile Fmoc-group and to avoid formation of by-products. Also, benzyl group of benzyl-L-aspartic acid should be intact throughout the process of SPPS. The *t*-butyl group has been the choice of proper protecting group to satisfy such requirements, because it is base-stable and can be simultaneously removed during the mild acid (TFA) treatment for the cleavage of the resin in the last step of synthesis.

Table I. Yields, retention times, and R_f values of the six decapeptide derivatives

Compound ID	Yield (%)	Retention Time (min)[a]	R_f[b]
A2	>95	6.89	0.60
A3	>95	4.41	0.64
A4	90	4.69	0.63
F2	95	7.82	0.38
F3	70	7.07	0.37
F4	86	6.56	0.38

[a] Column: Xterra@ MS C_{18} 3.5 μm, 2.1×150 mm; mobile phase: mixture of aqueous solution of 50 mM ammonium formate and acetonitrile (98:2, v/v); detector: UV at 254 nm; flow rate: 150 μL/min; injection volume: 3 μL

[b] TLC: On silica gel plate with mobile phase of DCM and MeOH (2:1, v/v)

A2 : (Asp-4-Bn)-(Asp-4-Bn)-$(Asp)_7$-Asp-OH
A3 : (Asp-4-Bn)-$(Asp\text{-}4\text{-}Bn)_2$-$(Asp)_6$-Asp-OH
A4 : (Asp-4-Bn)-$(Asp\text{-}4\text{-}Bn)_3$-$(Asp)_5$-Asp-OH
F2 : (Asp-4-Bn)-$(Asp)_7$-(Asp-4-Bn)-Asp-OH
F3 : (Asp-4-Bn)-$(Asp)_3$-(Asp-4-Bn)-$(Asp)_3$-(Asp-4-Bn)-Asp-OH
F4 : (Asp-4-Bn)-(Asp)-(Asp-4-Bn)-$(Asp)_2$-(Asp-4-Bn)-$(Asp)_2$-(Asp-4-Bn)-Asp-OH

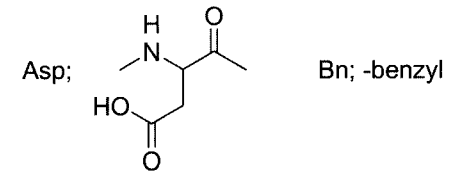


Fig. 2. Six decapeptide derivatives bearing the benzyl groups oat different positions.

The methodology resulted in relatively high yield (70-95 %) of the new target compounds as shown in Table I. We have synthesized six decapeptides represented as A2, A3, A4, F2, F3, and F4, where the first letter depicts the location of benzyl-L-aspartic acid residues either adjacent to (A) or far apart (F) from each other and numeric number represents the number of benzyl-L-aspartic acid residues present in the decapeptide (Fig. 2). Thin layer chromatography of each decapeptide revealed a single peak after UV detection at 254 and 351 nm. Purity of the product was further verified by HPLC/MS equipped with UV detector and electrospray ionization unit (Finnigan LCQ Advantage LC/MS/MS Spectrometer). The column and mobile phase were C_{18} reverse phase column (Xterra@ MS C_{18} 3.5 μm, 2.1×150 mm) and mixture of aqueous solution of 50 mM ammonium formate and acetonitrile (98:2 v/v), respectively. Flow rate was 150 μL / min and injection volume was 3 μL.

Fig. 3(a) shows a typical HPLC/UV chromatogram of compound A4 at 254 nm. MS spectrum of the compound showed multiple peaks in addition to the peak corresponding to the molecular ion of the product. In electrospray ionization technique, it is not unusual to get a number of different ions with different charge states depending on the conformation of peptide chain (Chernushevich *et al.*, 2001; Mann *et al.*, 2001; Whitelegge *et al.*, 1998). Therefore, we did not attempt at this stage to explain the formations of fragments and adducts of each decapeptide. Instead, we set the *m/z* of mass chromatogram at 1530 ($[M+1]^+$ for A4) and tried to identify if the peak occurs at the same retention time. The mass chromatogram selected at *m/z* of 1530 demonstrated a single peak at the same retention time, indicating that the single peak is from the decapeptide A4 (Fig. 3(b)). Five other decapeptides were identified in a similar way.

Together with the evidences from TLC and HPLC/MS, it was confirmed that the decapeptide products were reasonably pure and there were no significant contaminants or impurities, which is a unique characteristic of the SPPS methodology. Retention time of each decapeptide in HPLC is shown in Table I. The difference in retention time of each product may be attributed to the different degrees of polarities and hydrophobicities introduced to the compounds. Retention times of the decapeptides of A-series and F-series were around 4 and 7 min, respectively. Interestingly, retention time of F-series compounds was the opposite of the expectation, showing shorter retention time for the compound with more benzyl groups. Furthermore, the retention time of A2 exhibited delay of more than 2 min when compared with A3 and A4. This unexpected behavior is currently being studied in our lab with respect to the conformational alignment of benzyl and carboxylic groups in the compound.

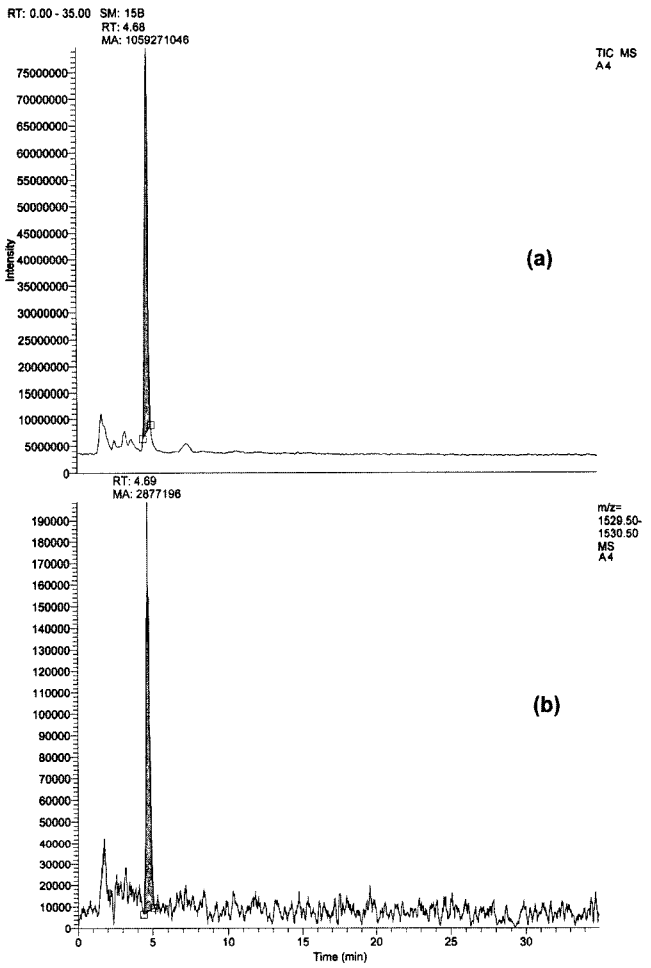


Fig. 3. Typical HPLC chromatogram and selected mass chromatogram of compound A4. (a): HPLC/UV chromatogram of compound A4 at detection wavelength of 254 nm, (b) selected mass chromatogram of compound A4 at m/z of 1530.

We have successfully prepared the decapeptides consisting of L-aspartic acid and benzyl-L-aspartic acid with

two, three, and four benzyl groups either adjacent to or far apart from each other. This methodology could be utilized for tailoring other polypeptides substituted with different numbers of benzyl groups, as per the requirement. The effect of the decapeptides comprising of L-aspartic acid and benzyl-L-aspartic acid on the reduction of AmB toxicity is in progress.

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REFERENCES

Berman, J. D., Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin. Infect. Dis.,* 24, 684-703 (1997).

Bodey, G. P., Anaissie, E. J., Elting, L. S., Estey, E., O'Brien, S., and Kantarjian, H., Antifungal prophylaxis during remission induction therapy for acute leukemia: fluconazole versus intravenous amphotericin B. *Cancer,* 73, 2099-2106 (1994).

Borowski, E., Novel approaches in the rational design of antifungal agents of low toxicity. *Farmaco,* 55, 206-208 (2000).

Brajtburg, J., Powderly, W. G., Kobayashi, G. S., and Madoff, G., Amphotericin B: delivery systems. *Antimicrob. Agents Chemother.*, 34, 381-384 (1990).

Branch, R. A., Prevention of amphotericin B-induced renal impairment: a review on the use of sodium supplementation. *Arch. Intern. Med.,* 148, 2389-2394 (1988).

Brime, B., Molero, G.., Frutos P., and Frutos, G.., Comparative therapeutic efficacy of a novel lyophilized amphotericin B lecithin-based oil-water microemulsion and deoxycholate-amphotericin B in immunocompetent and neutropenic mice infected with Candida albicans. *Eur. J. Pharm. Sci.,* 22, 451-458 (2004).

Carpino, L. A. and Han, G.. Y., 9-Fluorenylmethoxycarbonyl amino-protecting group. *J. Org. Chem.,* 37, 3404-3409 (1972).

Chernushevich, I. V., Loboda, A. V., and Thomson, B. A., An introduction to quadrupole–time-of-flight mass spectrometry. *J. Mass Spectrom.,* 36, 849–865 (2001).

Kaloyanides G. J., Antibiotic-related nephrotoxicity. *Nephrol. Dial. Transplant.,* 9 (suppl 4), 130-134 (1994).

Kishore, B. K., Kallay Z, Lambricht P, Laurent G, and Tulkens P. M., Mechanism of protection afforded by polyaspartic acid against gentamycin-induced phospholipidosis I. Polyaspartic acid binds gentamicin and displaces it from negatively charged phospholipid layers *in vitro. J. Pharmacol. Exp. Ther.,* 255, 867-874 (1990a).

Kishore, B. K., Lambricht, P., Laurent, G., Maldague, P., Wagner, R., and Tulkens, P. M., Mechanism of protection afforded by polyaspartic acid against gentamicin-induced phospholipidosis II. Comparative *in vitro* and *in vivo* studies with poly-L-aspartic, poly-L-glutamic, and poly-D-glutamic acids. *J. Pharmacol. Exp. Ther.,* 255, 875-885 (1990b).

Mann, M., Hendrickson, R. C., and Pandey, A., Analysis of proteins and proteomes by mass spectrometry. *Annu. Rev. Biochem.,* 70, 437-473 (2001).

Merrifield, R. B., Solid phase peptide synthesis: the synthesis of a tetrapeptide. *J. Am. Chem. Soc.,* 85, 2149-2154 (1963).

Meyer, R. D., Young, L. S., Armstrong, D., and Yu, B., Aspergillosis complicating neoplastic disease. *Am. J. Med.,* 54, 6-15 (1973).

Miranda, L. P. and Alewood, P. F., Accelerated chemical synthesis of peptides and small proteins. *Proc. Natl. Acad. Sci. U.S.A.,* 96, 1181-1186 (1999).

Mullen, A. B., Carter, K. C., and Baillie, A. J., Comparison of the efficacies of various formations of amphotericin B against murine visceral leishmaniasis. *Antimicrob. Agents Chemother.,* 41, 2089-2092 (1997).

Saxena, S. and Ghosh, P. C., Biodistribution of amphotericin B when delivered through cholesterol hemisuccinate vesicles in normal and A. fumigatus infected mice. *Pharm. Res.*, 17, 1236-1242 (2000).

Sheppard, R. C., Modern methods of solid-phase peptide synthesis. *Science Tools,* 33, 122-129 (1986).

Townsend, R. W., Zutshi, A., and Bekersky, I., Biodistribution of 4-[(14) C] cholesterol-Ambisome following a single intravenous administration to rats. *Drug Metb. Dispos.,* 29. 681-685 (2001).

Warnock, D. W., Amphotericin B: an introduction. *J. Antimicrob. Chemother.,* 28(Suppl. B), 27-38 (1991).

Wiebe, V. J. and DeGregorio, M. W., Liposome-encapsulated amphotericin B: a promising new treatment for disseminated fungal infections. *Rev. Infect. Dis.,* 10, 1097-1101 (1988).

Whitelegge, J. P., Gundersen, C. B., and Faull, K. E., Electrospray-ionization mass spectrometry of intact intrinsic membrane proteins. *Protein Science,* 7, 1423-1430 (1998).