Correlation of Antifungal Activity with Fungal Phospholipase Inhibition Using a Series of Bisquaternary Ammonium Salts

Clarissa K. L. Ng,[‡] Daniel Obando,[‡] Fred Widmer,[§] Lesley C. Wright,[§] Tania C. Sorrell,[§] and Katrina A. Jolliffe*[,‡]

School of Chemistry, The University of Sydney, 2006, NSW, Australia, and Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, and the Centre for Clinical Research Excellence in Infections, Bioethics and Haematological Malignancies, The University of Sydney at Westmead, Westmead, 2145, NSW, Australia

Received September 6, 2005

A series of bisquaternary ammonium salts with a 12-carbon spacer between the positive charges were synthesized, and their antifungal activity has been investigated. Compounds with butyl, pentyl, and isopentyl headgroups were the most potent antifungal agents with MICs in the range of 2.2−5.5 μM against both *Cryptococcus neoformans* and *Candida albicans*. The antifungal activity of these compounds correlated with their inhibition of cryptococcal phospholipase B1 (PLB1), a newly identified virulence factor. This indicates that the mode of action of these compounds may be inhibition of the fungal PLB1 enzyme, further validating this enzyme as a target for the development of novel antifungal therapies.

Introduction

Invasive fungal infections (mycoses) are becoming increasingly implicated as a cause of serious and fatal diseases. This is especially the case in immunocompromised patients, who are prone to infections caused by opportunistic fungal pathogens that are normally kept in check by a functioning immune system.[1,2] Despite recent additions of new classes of antifungal agents (e.g. caspofungin),[3] the number of currently available drugs for treatment of fungal infections is limited. Many of these are fungistatic (inhibit fungal growth) rather than fungicidal, while fungicidal drugs such as amphotericin B are toxic.[4] The emergence of fungi resistant to these drugs is also becoming problematic. It is now widely recognized that there is a need for the development of new antifungal drugs that have a different mode of action to those currently in use.[5]

Virulence factors and biochemical and signal transduction pathways unique to fungi are potential targets for antifungal drug development. Recently, secreted enzymes termed phospholipase B (PLB1) have been identified as virulence determinants of the pathogenic fungi, *Candida albicans* and *Cryptococcus neoformans*.[6−9] The secretion of PLB1 by other pathogenic fungi (including *Aspergillus sp.*) has also been detected.[10] PLB1 has been purified from *C. neoformans* and characterized as a single protein containing three separate activities.[9,11] These include phospholipase B (PLB), which removes both acyl chains simultaneously from phospholipids; lyso-phospholipase (LPL), which removes the single acyl chain from lysophospholipids; and LPL transacylase (LPTA), which adds an acyl chain to lysophospholipids to form phospholipids (Figure 1).

The structure and mechanism of action of PLB1 have not been determined, and which of the secreted phospholipase activities is important in virulence is unknown. However, PLB1 has a role in the survival and replication of *C. neoformans* within macrophages,[11] and in the destruction of lung tissue and the production of eicosanoids, which modulate phagocytic activity.[12] This, together with the apparently unique gene sequence and properties of PLB1s from pathogenic fungi define them as potential targets for antifungal therapy.

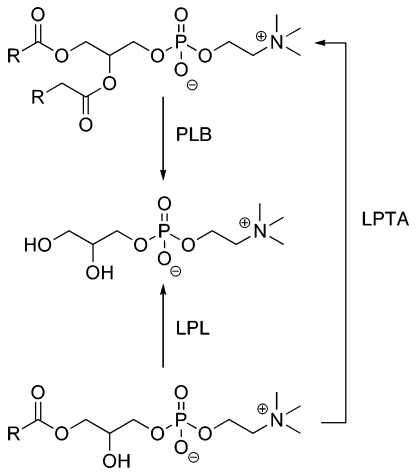


Figure 1. The three activities of cryptococcal phospholipase PLB1.

Hänel et. al. have reported that several beta-blocker-like compounds both inhibit secreted fungal phospholipases (as detected by egg yolk agar assays) and exhibit antifungal activity.[13] However, the assays employed did not allow the type of phospholipase that was inhibited to be determined. More recent work by Ganendren et. al. described a novel class of phospholipase inhibitors and established that compounds that inhibited cryptococcal PLB1 also exhibited antifungal activity in a standard broth microdilution susceptibility assay.[14] Intriguingly, 1,12-bis(tributylphosphonium)dodecane dibromide (**1**) was found to exhibit moderate PLB inhibition and antifungal activity, while the structurally similar 1,10-bis(trimethylammonium)decane (decamethonium, **2**) was not inhibitory toward fungal PLB1 and was not antifungal.

These observations prompted us to synthesize a series of structurally related bis(quaternary ammonium)alkanes (**3**−**14**) with a 12-carbon spacer between the positive charges and to use these compounds as tools to investigate the validity of cryptococcal PLB1 as a novel target for antifungal therapy.

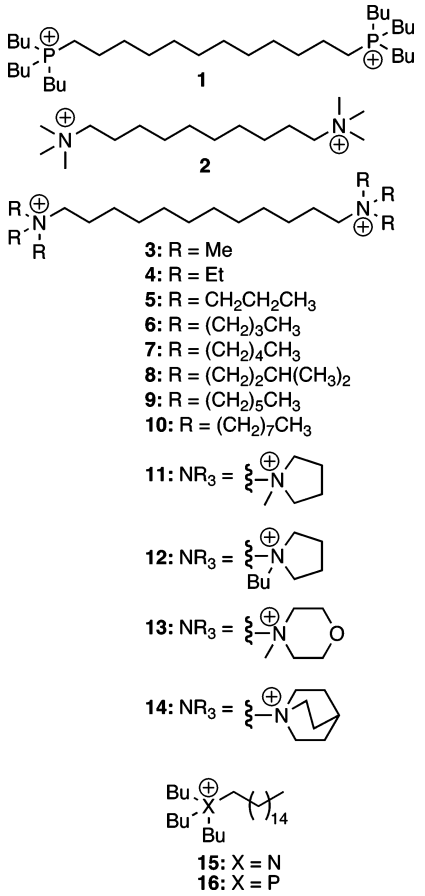
* To whom correspondence should be addressed. Tel: +61 2 9351 2297; Fax: +61 2 9351 3329; e-mail: jolliffe@chem.usyd.edu.au.

[‡] School of Chemistry, The University of Sydney.

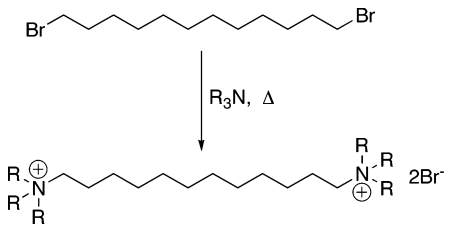
[§] Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, and the Centre for Clinical Research Excellence in Infections, Bioethics and Haematological Malignancies, The University of Sydney at Westmead.

10.1021/jm0508843 CCC: $33.50
Published on Web 12/16/2005

Chart 1. Structures of the Mono- and Bisquaternary Ammonium and Phosphonium Compounds (counterions not specified − See Table 1)



Scheme 1. Synthesis of the Bisquaternary Ammonium Salts



Results

Chemistry. The bis(quaternary ammonium) salts were, in most cases, readily prepared by treatment of the appropriate dibromide with an excess of trialkylamine in the absence of oxygen (Scheme 1). For headgroups bearing longer alkyl chains (e.g. **9**, **10**), the yields were notably lower and longer reaction times were required. Where the products were solids, they were purified by recrystallization as the dibromide salts. However, many of the compounds were isolated as viscous oils or waxes. These were purified by repeated trituration or column chromatography on silica gel, followed by ion exchange to give the dichloride salts.[15]

Antifungal Activity. Compounds **3**−**14** were assayed by a standardized serial dilution sensitivity test for their antifungal activities against reference strains of *Cryptococcus neoformans* and *Candida albicans* (Table 1). The most potent compounds against both *Candida* and *Cryptococcus* species were those bearing the tributyl, tripentyl, and triisopentyl headgroups, with only small differences in activity observed for these three compounds. For the linear alkane series, the MIC drops sharply upon increasing the headgroup bulk from methyl to ethyl and continues to drop as the length of the alkyl chains is extended to reach a minimum at butyl (for *Cryptococcus*) or pentyl (for *Candida*). Further lengthening the headgroup chains leads to a decrease in antifungal activity. Notably, the tributylphosphonium (**1**) and tributylammonium (**6**) analogues had essentially identical activities. Branching at the 3-position of the chains in the isopentyl derivative (**8**) does not significantly alter the antifungal activity of the compounds compared with the straight chain butyl (**6**) or pentyl (**7**) analogues. However, the incorporation of heterocyclic headgroups (compounds **11**−**14**) results in little or no antifungal activity. Changing the counterions from bromide to chloride did not appear to have any effect on antifungal activity.

The most active compound, **6**, was assayed against a broad range of yeasts and filamentous fungi (Table 2). While **6** is slightly less potent than the broad-spectrum antifungal drug amphotericin B, it shows a remarkable range of activity.

Phospholipase Inhibition. The compounds were assayed against both cryptoccocal PLB1 and porcine pancreatic PLA_2 ($ppPLA_2$). The inhibition assays against the secreted cryptococcal activities of a crude supernatant were carried out at pH 4, under conditions previously optimized to measure PLB, LPL, and LPTA activities against the secreted form of the PLB1 multifunctional enzyme.[14] All compounds were initially tested at concentrations of 250, 25, 2.5, and 0.25 μM. None of the compounds strongly inhibited the LPL/LPTA activities of the enzyme at any concentration tested, but compounds **5**−**9** exhibited significant inhibition of the PLB activity with an IC_{50} range of 3−10 μM (Table 1). Notably, these compounds also exhibited the strongest antifungal activity, while compounds **3**, **11**, and **14**, which were not antifungal, did not show any inhibition of PLB1 activity (Figure 2). This indicates a tight correlation between enzyme inhibition and antifungal activity.

The inhibition assays against $ppPLA_2$ were carried out according to the method of De Haas et al.[16] Compounds **3** and **6**−**9** were tested at concentrations of 250, 25, 2.5, and 0.25 μM. Compounds **6**−**9**, which showed substantial inhibition of PLB1 (Figure 2), inhibited $ppPLA_2$ in the range of 90−99% at 250 μM, but showed 0% inhibition of $ppPLA_2$ at concentrations of 25 μM and below. Compound **3**, which did not substantially inhibit PLB1 (Figure 2), showed no inhibition of PLA_2 at any concentration. Thus, since compounds **6**−**9** inhibit fungal PLB1 much more strongly than $ppPLA_2$, it is possible to achieve a high degree of selectivity between these two enzymes.

Hemolytic Assays. Since a number of the bisquaternary ammonium salts exhibit strong antifungal activity and selectivity for the fungal over the mammalian enzyme, they have potential to be developed as antifungal therapeutics. However, the structures of these compounds are similar to those of bolaform surfactants and, as such, it was thought that they might have a simple lytic mode of action and be broadly cytotoxic against human cells. We therefore determined the hemolytic activity of these compounds as a measure of their cytotoxicity and an initial test of their suitability for further development.

Compounds **2**, **3**, and **12** bearing short and/or heterocyclic trialkyl headgroups did not lyse erythrocytes at any of the concentrations examined (Table 3). For the strongly antifungal compounds **6** and **7**, hemolysis at concentrations well above the MICs, e.g. 175 μM, is only 5%, while for the triisopentyl derivative, **8**, hemolysis is 30% at this concentration, but drops to 4% at 87.5 μM, which is still well above the MICs.

For comparison, we prepared and tested the hemolytic activity of hexadecyltributylammonium bromide (**15**). This monoqua-

Table 1. Influence of Bisquaternary Ammonium Head Group Structure on in Vitro Antifungal Potency and Inhibition of Secreted Cryptococcal PLB Activity

Compound	Headgroup	X^-	MIC (μM)		PLB IC_{50} (μM)
			C. neoformans (ATCC 90112)	*C. albicans* (ATCC 10231)	
1	$-^+PBu_3$	Br	2.5[a]	8.8[a]	> 25[c]
2	$-^+NMe_3$	Br	≥ 350[a]	≥ 350[a]	> 250[b]
3	$-^+NMe_3$	Br	175	175	> 250[b]
4	$-^+NEt_3$	Br	11	22	> 250[b]
5	$-^+NPr_3$	Br	5.5	10	7.0[c]
6	$-^+NBu_3$	Cl[d]	2.2	5.5	10.0[c]
7	$-^+N[(CH_2)_4CH_3]_3$	Br	5.5	5.5	3.5[c]
8	$-^+N[(CH_2)_2CH(CH_3)]_3$	Cl	4.4	2.2	7.5[c]
9	$-^+N[(CH_2)_5CH_3]_3$	Cl	17.5	8.8	7.5[c]
10	$-^+N[(CH_2)_7CH_3]_3$	Cl	35	100	> 250[b]
11		Br	175	175	> 250[b]
12		Cl	88	88	> 250[b]
13		Br	44	88	> 250[b]
14		Br	350	≥ 350	> 250[b]

[a] The activity of these compounds has already been reported.[14] [b] Inhibition was determined for all compounds at 250 μM, 25 μM, 2.5 μM, and 0.25 μM concentrations. These compounds showed no significant inhibition of any of the three activities of cryptococcal PLB1 (see Figure 2). [c] IC_{50} values for PLB were determined using a concentration range of 250 μM, 25 μM, 12.5 μM, 6.25 μM, 3.13 μM, 1.6 μM, and 0.25 μM. None of the compounds tested showed more than 20% inhibition of the LPL and LPTA activities of PLB1 at the highest concentration of 250 μM. [d] Identical MIC values were obtained for compound **6** when the counterion was Br^-.

Table 2. In Vitro Antifungal Activity Spectrum of 1,12-Bis(tributylammonium)dodecane Dichloride (**6**) Compared with Amphotericin B

	MIC (μM)	
	6[a]	amphotericin B[b]
Aspergillus fumigatus ATCC 204 305	11	0.54
Aspergillus terreus ATCC 03−232−378	5.5	1.1
Aspergillus flavus ATCC 204 304	22	0.54
Scedosporium prolificans 1−003−040	5.5	4.3
Scedosporium apiospermum 1−003−056	2.8	0.54
Fusarium solani 04−132−4207	5.5	0.27
Cryptococcus neoformans ATCC 90112	2.2	0.54
Candida albicans ATCC 10231	5.5	0.54

[a] Molecular weight 608.6. [b] Molecular weight 924.1.

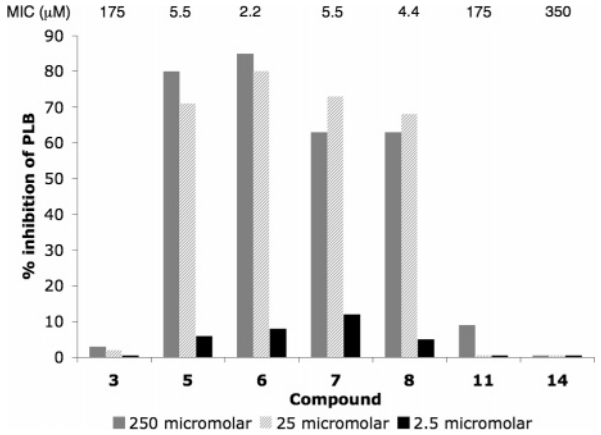


Figure 2. Percentage inhibition of secreted cryptococcal PLB activity at compound concentrations of 250, 25, and 2.5 μM. MIC values for *C. neoformans* are also given, illustrating the correlation between in vitro antifungal activity and PLB inhibition.

ternary ammonium salt is considerably more hemolytic than all of the bisquaternary ammonium salts we have tested, retaining a significant residual hemolytic activity of 2.5% at the lowest concentration (3.5 μM) tested (Table 3). Similarly, the commercially available hexadecyltributylphosphonium bromide (**16**) retains 2.5% hemolytic activity at this concentration.

Table 3. Hemolytic Activity of Mono- and Bisquaternary Ammonium and Phosphonium Compounds

	percentage lysis of human red blood cells as a function of compound concentration				
compound	350 μM	175 μM	87.5 μM	35 μM	3.5 μM
1	15	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
6	100	5	0	0	0
7	100	5	0	0	0
8	100	30	4	0	0
12	0	0	0	0	0
15	100	75	9	4	2.5
16	100	32	7	4	2.5

Discussion

Fungal phospholipases are an emerging new target for antifungal therapy.[6−9,17] However, relatively few inhibitors of these enzymes have been reported and their precise role in pathogenicity is still being investigated. A preliminary study indicated that the ion-pairing agent **1** both inhibits cryptococcal PLB1 activity and is antifungal, while the structurally similar decamethonium **2** is neither inhibitory nor antifungal.[14] In the present study, a series of bisquaternary ammonium salts have been synthesized and structure−activity relationship studies have indicated that their antifungal and PLB1 inhibitory activities are correlated.

The most active compounds **6**−**8** have tributyl-, tripentyl-, and tri(isopentyl)- headgroups and MICs of 2.2−5.5 μM against representative strains of both *C. neoformans* and *C. albicans* species. In addition, compound **6** shows a broad spectrum of activity against both yeasts and filamentous fungi, including some resistant species and species of emerging clinical significance. The same compounds have IC_{50} values of 3.5−10 μM against the PLB activity of the multifunctional cryptococcal PLB1, while compounds (e.g. **3**, **11**, and **14**) that do not exhibit antifungal activity show little or no inhibition of the activities of cryptococcal PLB1. This indicates that antifungal activity is correlated with PLB1 inhibition and further validates this enzyme as a novel target for antifungal therapy.

The PLB1 inhibitors show some selectivity for fungal PLB1 over a representative mammalian PLA ($ppPLA_2$) with compound **7** having an IC_{50} of 3.5 μM against the PLB activity of cryptococcal PLB1 and no inhibition of $ppPLA_2$ at more than seven times this concentration (25 μM). The broader cytotoxicity profile of this class of compounds is yet to be established. However, it is clear that these bisquaternary ammonium salts have surprisingly low lytic properties, despite their structural similarities to the bolaform surfactants. Compounds **6** and **7** show no hemolytic activity at concentrations 40−80-fold greater than the MIC.

Conclusions

The synthesis of a series of bisquaternary ammonium salts has resulted in the discovery of a novel class of antifungal agents with a broad spectrum of activity. The antifungal activity of these compounds correlated with their inhibition of fungal PLB1, suggesting that this may be their mode of action and further validating this enzyme as a target for the development of novel antifungal therapies. Current work in our laboratories is focused on improving both the potency and selectivity of this promising class of antifungal agents. In addition, we are carrying out structure−activity relationship studies and examining the structure of cryptococcal PLB1 to further elucidate the relationship between antifungal activity and PLB inhibition.

Experimental Section

Chemistry. Melting points were determined using a Gallenkamp melting point apparatus and are reported in degrees Celsius (uncorrected). NMR spectra were recorded on a Bruker Avance DPX 200 or a Bruker Avance DPX 300 spectrometer. The solvent ^1H and ^{13}C signals, δ_H 7.26 for residual $CHCl_3$ and δ_C 77.0 for $CDCl_3$, δ_H 3.31 and δ_C 49.0 for d_4-MeOH, δ_H 2.50 and δ_C 39.5 for d_6-DMSO, were used as internal references. Low resolution mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer (ESI). High-resolution mass spectra were recorded on a BioApex Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (ESI). Flash chromatography was performed on silica gel (Merck silica gel 60, 40−63 μm) at a pressure of 0.3−0.4 bar. Ion exchange chromatography was performed on Lewatit MP-64 resin (chloride form), which was swollen in H_2O, washed with three bed volumes of 0.1 M HCl (aq), and then washed with H_2O until the washings were neutral. Elemental analyses were performed by Campbell Microanalytical Laboratories. Compounds **1**, **2**, and **16** were obtained from Sigma-Aldrich Chemical Co. Compounds **3**, **4**, and **11** were prepared according to the method of Calas et al.[18,19] Compound **15** was prepared according to the method of Shelton et al.[20]

General Procedure for the Synthesis of Bisquaternary Ammonium Salts. Trialkylamine (excess) was added to a solution of dibromoalkane either neat or in methylisobutyl ketone, and then the mixture was deoxygenated using a freeze−thaw process and heated at 100 °C under a nitrogen atmosphere for 48 h. The resulting mixture was cooled to room temperature. If no precipitate formed upon cooling, excess solvent was removed under reduced pressure and the residue purified as described below.

1,12-Bis(tripropylammonium)dodecane Dibromide (5). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with tripropylamine (1.72 g, 12.1 mmol) in methyl isobutyl ketone (2 mL) according to the general method. The resulting brown oil was triturated with anhydrous diethyl ether (3 × 10 mL) and then with anhydrous ethyl acetate (3 × 10 mL) and dried under vacuum to yield **3** as a pale yellow oil (0.70 g, 75%). ^1H NMR (200 MHz, CD_3OD): δ 3.32 (16H, m), 1.75 (16H, m), 1.40 (16H, m), 1.04 (18H, t, J 7.2 Hz). ^{13}C NMR (300 MHz, CD_3OD): δ 61.7, 60.3, 31.1, 30.7, 27.9, 23.3, 16.8, 11.3, 1 signal obscured or overlapping. MS (ESI) m/z 227 $[M - 2Br^-]^{2+}$ (100). Found $[M - 2Br^-]^{2+}$ 227.4287, $[C_{30}H_{66}N_2]^{2+}$ 227.4287.

1,12-Bis(tributylammonium)dodecane Dichloride (6). 1,12-Dibromododecane (1.0 g, 3.05 mmol) was treated with tributylamine (1.13 g, 6.09 mmol) in methyl isobutyl ketone (2 mL) according to the general method. The resulting brown oil was purified by flash chromatography, eluting with 90:10:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.20). The fractions containing the required compound were then combined, and the solvent was removed under reduced pressure. The residue was dissolved in the minimum amount of H_2O and passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with H_2O. Concentration of the appropriate fractions gave **6** as a pale yellow oil (0.5 g, 27%). ^1H NMR (300 MHz, CD_3OD): δ 3.29 (16H, m), 1.65 (16H, br. s), 1.47−1.34 (28H, br. m), 1.01 (18H, t, J 7.1 Hz). ^{13}C (300 MHz, CD_3OD): 59.1, 58.5, 29.7, 29.2, 26.4, 23.8, 21.8, 19.7, 12.9, 1 signal obscured or overlapping. MS (ESI) m/z 241 $[M - 2Cl^-]^{2+}$ (100%), 573 $[M - Cl^-]^+$ (77). Found $[M - Cl^-]^+$ 573.5865, $[C_{36}H_{78}N_2Cl]^+$ requires 573.5852. Anal. ($C_{36}H_{78}Cl_2N_2 \cdot 3.5H_2O$) calcd, C 64.25; H 12.7; N 4.2. Found C 64.6; H 12.4; N 4.2.

1,12-Bis(tripentylammonium)dodecane Dibromide (7). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with tripentylamine (1.38 g, 6.08 mmol) according to the general method. The resulting brown oil was triturated with anhydrous diethyl ether (3 × 10 mL), and the residue was dried under vaccum to yield **7** as a brown oil (0.95 g, 80%). ^1H NMR (300 MHz, CD_3OD): δ 3.30 (16H, m), 1.67 (16H, m), 1.39 (40H, m), 0.96 (18H, t, J 7.2 Hz). ^{13}C (300 MHz, CD_3OD): δ 58.7, 29.7, 29.3, 28.5, 26.4, 22.2, 21.8, 21.5 13.2, two signals obscured or overlapping. MS (ESI) m/z 311 $[M - 2Br^-]^{2+}$ (100%), 703 $[M - Br^-]^+$ (76). Found $[M - 2Br^-]^{2+}$ 311.3541, $[C_{42}H_{90}N_2]^{2+}$ requires 311.3547.

1,12-Bis(triisopentylammonium)dodecane Dichloride (8). 1,12-Dibromododecane (1.0 g, 3.05 mmol) was treated with triisopentylamine (0.78 g, 3.42 mmol) in methyl isobutyl ketone (2 mL) according to the general method. The resulting brown oil was purified by flash chromatography, eluting with 90:10:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.20). The fractions containing the required compound were then combined and the solvent removed under reduced pressure. The residue was dissolved in the minimum amount of EtOH and passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with 3:7 EtOH/H_2O. Concentration of the appropriate fractions gave **8** as a pale yellow oil (0.37 g, 31%). ^1H NMR (300 MHz, CD_3OD): δ 3.32 (16H, m), 1.72 (16H, m), 1.60 (6H, m), 1.06 (16H, m), 1.03 (36H, d, J 6.5 Hz). ^{13}C (300 MHz, CD_3OD): δ 58.4, 57.3, 30.2, 29.7, 29.6, 29.2, 26.5, 26.3, 21.7, one signal obscured or overlapping. MS (ESI) m/z (before ion exchange) 703 $[M - Br^-]^+$ (45). Found $[M - Br^-]^+$ 703.6272, $[C_{42}H_{90}N_2Br]^+$ requires 703.6267. Anal. ($C_{42}H_{90}Cl_2N_2 \cdot 3.5H_2O$) Calcd C 66.6; H 12.9; N 3.7. Found C 66.7; H 12.5; N 3.95.

1,12-Bis(trihexylammonium)dodecane Dichloride (9). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with trihexylamine (0.82 g, 3.05 mmol) in methyl isobutyl ketone (2 mL) according to the general method. The resulting brown oil was purified by flash chromatography, eluting with 90:10:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.20). The fractions containing the required compound were then combined, and the solvent removed under reduced pressure. The residue was dissolved in the minimum amount of EtOH and passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with 3:7 EtOH/H_2O. Concentration of the appropriate fractions gave **9** as a pale yellow oil (0.14 g, 12%).^1H NMR (200 MHz, CD_3OD): δ 3.34 (16H, m), 1.78−1.65 (16H, m), 1.42 (52H, m), 0.97 (18H, m). ^{13}C (300 MHz, CD_3OD): δ 58.8, 31.5, 29.8, 26.2, 22.6, 21.9, 13.4, five signals obscured or overlapping. MS (ESI) m/z 354 $[M - 2Cl^-]^{2+}$ (100%), 743 $[M - Cl^-]^+$ (45). Found: $[M - 2Cl^-]^{2+}$ 353.6547, $[C_{48}H_{102}N_2]^{2+}$ requires 353.6547.

1,12-Bis(trioctylammonium)dodecane Dichloride (10). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with trioctylamine (1.12 g, 3.17 mmol) in methyl isobutyl ketone (2 mL) according to the general method. The resulting brown oil was purified by flash chromatography, eluting with 90:10:1 CH_2Cl_2/MeOH/$NH_{3(aq)}$ (R_f 0.20). The fractions containing the required compound were then combined, and the solvent was removed under

reduced pressure. The residue was dissolved in the minimum amount of EtOH and passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with 3:7 EtOH/H_2O. Concentration of the appropriate fractions gave **10** as a pale yellow oil (0.11 g, 7%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.37−3.19 (16H, m), 1.78−1.65 (16H, m), 1.32−1.23 (76H, m, CH_2), 0.89−0.82 (18H, t, *J* 7.1 Hz). ^{13}C (300 MHz, CD_3OD): δ 58.8, 32.1, 29.9, 29.8, 29.4, 29.3, 26.5, 22.9, 22.0, 13.6, 4 signals obscured or overlapping. MS (ESI) *m*/*z* 466 $[M - 2Cl^-]^{2+}$ (100%), 909 $[M - Cl^-]^+$ (50). Found: $[M - Cl^-]^+$ 909.9603, $[C_{60}H_{126}N_2Cl]^+$ requires 909.9610.

1,12-Bis(*N*-butylpyrrolidinium)dodecane Dichloride (12). 1,12-Dibromododecane (0.5 g, 1.52 mmol) was dissolved in pyrrolidine (0.44 g, 6.08 mmol), and the resulting mixture was stirred at 90 °C for 20 h. The crude mixture was purified by flash chromatography, eluting with 80:18:2 CH_2Cl_2/MeOH/$NH_{3(aq)}$. Concentration of the appropriate fractions (R_f 0.17) gave 1,12-bispyrrolidine dodecane as a yellow solid (0.33 g, 71%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.51−2.36 (12H, m), 1.80−1.74 (8H, m), 1.53−1.47 (4H, m), 1.27−1.26 (16H, m). MS (ESI) *m*/*z* 310 $[M + H^+]^+$ (100).

To 1,12-bispyrrolidine dodecane (0.33 g, 1.08 mmol) in methyl isobutyl ketone (2 mL) was added butyl bromide (0.88 g, 6.46 mmol), and the resulting mixture was stirred at reflux for 20 h. The resulting brown oil was purified by flash chromatography, eluting with 80:18:2 CH_2Cl_2/MeOH/$NH_{3(aq)}$, (R_f 0.28). The fractions containing the required compound were then combined, and the solvent was removed under reduced pressure. The residue was dissolved in the minimum amount of EtOH and passed through a column of Lewatit MP-64 anion resin (Cl^-), eluting with 3:7 EtOH/H_2O. Concentration of the appropriate fractions gave **12** as a pale yellow oil (0.45 g, 70%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.54 (8H, m), 3.27 (8H, m), 2.18 (8H, m), 1.70 (8H, m), 1.46−1.34 (20H, m), 1.01 (6H, t, *J* 7.2 Hz). ^{13}C NMR (300 MHz, CD_3OD): δ 63.0, 60.1, 59.8, 29.6, 29.5, 29.2, 26.5, 25.2, 23.3, 21.9, 19.8, 12.9. MS (ESI) *m*/*z* 211 $[M - 2Cl^-]^{2+}$ (100). Found $[M - 2Cl^-]^{2+}$ 211.2295, $[C_{22}H_{46}N_2]^{2+}$ requires 211.2295.

1,12-Bis(*N*-methylmorpholinium)dodecane Dibromide (13). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with *N*-methylmorpholine (0.61 g, 6.08 mmol) according to the general method. Upon cooling, a precipitate formed. This was collected by filtration and recrystallized from methanol/diethyl ether to give compound **13** as a colorless, hygroscopic solid (0.52 g, 65%), mp 194−197 °C. ^{1}H NMR (200 MHz, CD_3OD): δ 4.10−4.09 (8H, m), 3.68−3.46 (12H, m, CH_2), 3.36 (6H, s), 2.08−2.05 (4H, br. s), 1.44−1.35 (16H, m). ^{13}C NMR (300 MHz, CD_3OD): δ 64.5, 54.5, 29.8, 29.7, 29.4, 26.8, 24.1, 22.2, one signal obscured or overlapping. MS (ESI) *m*/*z* 185 $[M - 2Br^-]^{2+}$ (100%), 451 $[M - Br^-]^+$ (25). Found $[M - 2Br^-]^{2+}$ 185.3109, $[C_{22}H_{46}N_2]^{2+}$ requires 185.3109.

1,12-Bis(quinuclidinium)dodecane Dibromide (14). 1,12-Dibromododecane (0.50 g, 1.52 mmol) was treated with quinuclidine (0.34 g, 3.0 mmol) in methyl isobutyl ketone (1 mL) according to the general method. The mixture was deoxygenated by freeze/thaw and stirred at reflux for 24 h, at which time precipitate was formed. The crude mixture was cooled and the resulting precipitate was collected by filtration. Recrystallization from methanol/methyl-isobutyl ketone gave the compound **14** as a colorless hygroscopic solid (0.45 g, 54%), mp 87−90 °C. ^{1}H NMR (300 MHz, d_6-DMSO): δ 3.36−3.28 (12H, m), 3.06 (4H, m), 2.04 (2H, m), 1.82 (12H, br. s), 1.59 (4H, m), 1.24 (16H, br. s). ^{13}C NMR (300 MHz, d_6-DMSO): δ 64.6, 54.5, 29.8, 29.7, 29.4, 26.8, 24.2, 22.2, 19.8. MS (ESI) *m*/*z* 195 $[M - 2Br^-]^{2+}$ (100%), 470 $[M - Br^-]^+$ (75). Found $[M - 2Br^-]^{2+}$ 195.1979, $[C_{26}H_{50}N_2]^{2+}$ requires 195.1982.

Fungal Isolates and Media. A virulent clinical isolate of *C. neoformans* var. *grubii* (serotype A), H99, which produces high levels of secreted phospholipase B activity was used for cell-associated phospholipase characterization and inhibition of phospholipase activities. Isolate H99 was kindly supplied by Dr. Gary Cox (Duke University Medical Center, Durham, NC) and subcultured onto Sabouraud dextrose agar (SDA) at 30 °C.

Preparation of Supernatants Containing Secreted Phospholipase Activities. Isolate H99 was grown to confluence on SDA in 16 cm diameter Petri dishes for 72 h at 30 °C in air. Cells scraped from 10 to 20 dishes were washed sequentially with isotonic saline and imidazole buffer (10 mM imidazole, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 56 mM D-glucose, made up in isotonic saline, pH 5.5), resuspended in a volume of this buffer of about 10% of the cell volume, and incubated for 24 h at 37 °C. The cell-free supernatant was separated by centrifugation as previously described[21] and stored at −70 °C.

Radiometric Assay Method for Fungal Phospholipases. Enzyme activities were measured as described previously[8,11,21] in a final volume of 125 μL at 37 °C. For the determination of secreted PLB activity, carrier dipalmitoyl phosphatidylcholine (DPPC, final concentration 800 μM) and 1,2-di[1-^{14}C] palmitoyl phosphatidylcholine (20000 dpm) were dried under nitrogen and suspended in 125 mM imidazole acetate buffer (assay buffer, pH 4.0) by sonication using a Branson 450 sonifier. The reaction time was 22 min, using 1 μg total protein, and PLB activity was determined by the rate of decrease of the radiolabeled PC substrate, with appearance of the label in free fatty acid. Secreted LPL and LPTA activities were measured simultaneously in a reaction mixture containing 1-[-^{14}C]palmitoyl lyso-PC (25000 dpm) and carrier lyso-PC (final concentration 200 μM) in assay buffer. The reaction time was 15 s with 1 μg of total protein and LPL activity was measured by the rate of loss of 1-[-^{14}C]palmitoyl lyso-PC with release of radiolabeled fatty acids. LPTA activity was estimated from the rate of formation of radiolabeled PC. All reactions were terminated by adding 0.5 mL of chloroform:methanol (2:1 v/v). The reaction products were extracted by the method of Bligh and Dyer,[22] separated by TLC, and quantified as previously described.[21] The TLC plates were developed in chloroform:methanol:water (65:25:4; v/v/v).

Assay Method for Porcine Pancreatic PLA_2. Porcine pancreatic phospholipase A_2 was suspended in 3.2 M ammonium sulfate (2.9 mg protein/mL, Sigma St.Louis, MO). One part of well-mixed enzyme suspension was added to 4 parts of buffer [10 mM Tris/HCl, 10 mM $CaCl_2$, pH 8.2].[16] Activity and inhibition by test compounds were then measured by the radiometric method described above, except that 25 μL of enzyme solution was used, and the reaction time was 1 h. These conditions resulted in ∼60% substrate conversion in the inhibitor-free control.

Protein Assays. Total protein estimations were performed using a Coomassie Blue binding assay with BSA as standard (Pierce Chemical Co., Rockford, IL).

Testing of Bis-quaternary Ammonium Salts as Inhibitors. Solutions of bis-quaternary ammonium compounds were prepared as stock solutions of 700 μM in assay buffer containing 5 mM EDTA, which was then diluted serially with buffer to give solutions with concentrations of 70, 7, and 0.7 μM. In each assay, 45 μL of the stock or diluted solutions was used, and the final volume of 125 μL was made up of substrate, enzyme, and buffer. This gave solutions with compound concentrations of 250, 25, 2.5, and 0.25 μM. The radiometric assay was carried out as above. Inhibition was calculated as the percent of substrates (DPPC or Lyso-PC) remaining in the case of PLB and LPL activities, or of DPPC produced, in the case of the LPTA activity. The amounts converted, or produced, in the inhibitor-free control were normalized to 100%, and the inhibition was calculated against it. All assays were done in triplicate, with a difference between runs of <10% in all cases.

Antifungal Susceptibility Testing. The antifungal activity of the compounds was measured by standard broth microdilution methods of the US National Committee for Clinical Laboratory Standards for yeasts[23] and filamentous fungi.[24] The minimal inhibitory concentration (MIC) was defined as that which produced no visible growth after 48, or 72 h, of culture at 35 °C.[23,24] All tests were performed in duplicate.

Hemolytic Activity Assay. Human blood was collected in 10 mL Vacutainer tubes containing potassium-EDTA as anticoagulant. The blood from each Vacutainer was transferred to a 50 mL centrifuge tube, and the cells were washed three times with 30 mL

of calcium- and magnesium-free phosphate-buffered saline (PBS; Gibco). Cells were collected by centrifugation at 2000*g* for 10 min in a Beckman TJ-6 centrifuge. The third supernatant was clear and colorless. Cells were stored in PBS (20 mL) for up to two weeks. Then 0.5 mL cell suspension in PBS was mixed with (0.5 mL) of test substance using stock solutions of concentrations 700, 350, 175, 70, and 7 μM (final erythrocyte concentration around 0.5×10^9 per mL). The mixtures were incubated at 37 °C for 1 h with gentle shaking, centrifuged at 2000*g* for 10 min, the supernatant diluted 10-fold with PBS, and optical density measured at 540 nm. The values for 0% and 100% lysis were determined by incubating cells with PBS or 0.1% (w/v) Triton X-100 (in water), respectively. Assays were carried out in triplicate, and the difference between runs was <5% in all cases. The concentration of test compounds in the assays was 350, 175, 87.5, 35, and 3.5 μM.

Acknowledgment. This work was partly supported by the National Health and Medical Research Council of Australia project grant number 211040. K.A.J. thanks the Australian Research Council for the award of a Queen Elizabeth II research fellowship.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **5**−**10** and **12**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0508843