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Synthesis of a Highly Water-Soluble Derivative of Amphotericin B with Attenuated Proinflammatory Activity

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Abstract

Amphotericin B (AmB), a well-known polyene antifungal agent displays a marked tendency to self-associate and, as a consequence, exhibits very poor solubility in water. The therapeutic index of AmB is low, and is associated with significant dose-related nephrotoxicity, as well as acute, infusion-related febrile reactions. Reports in the literature indicate that that toxicity of AmB may be related to the physical state of the drug. Reaction of AmB in dimethylformamide with *bis*(dimethylaminopropyl)carbodiimide yielded an unexpected *N*-alkylguanidine/*N*-acylurea *bis*-adduct of AmB which was highly water soluble. The absorption spectrum of the AmB derivative in water indicated excellent monomerization, and the anti-fungal activities of reference AmB and its water-soluble derivative against *C. albicans* were found to be virtually identical. Furthermore, the water-soluble adduct is significantly less active in engaging TLR4 which would suggest that the adduct may be less proinflammatory.

Keywords

Amphotericin B; solubility; toxicity; aggregation; carbodiimide; antifungal; toll-like receptors

Introduction

Amphotericin B (AmB) is a polyene antifungal agent first isolated from *Streptomyces nodosus* in 1955 from Venezuelan soil samples near the Orinoco River region.[1] For more than a half-century, AmB has remained the cornerstone of the therapy of serious systemic fungal infections,[2;3] and even with the advent of alternate antifungal agents such as the triazoles (voriconazole,[4–6] and posaconazole[7;8]), and the echinocandins (caspofungin[9]), AmB is likely to remain an important therapeutic modality in the management of fulminant fungal infections.[10]

Amphotericin B, as the name suggests, is amphoteric, owing to the presence of carboxylate and amine functional groups and, is also amphipathic due to the asymmetric distribution of polar hydroxyl groups on face of the molecule, and a markedly hydrophobic, conjugated polyene hydrocarbon on the other (Fig. 1). AmB displays a marked tendency to self-associate with a critical aggregation concentration of ca. 0.2 μg/mL[11] and, as a consequence, exhibits very poor solubility in water (< 1 μg/mL), as well as in apolar organic solvents such as cyclohexane (20 μg/mL).[11] The therapeutic index of AmB is low, and is associated with significant dose-related nephrotoxicity,[12–14] as well as acute, infusion-related febrile reactions.[15;16] The unfavorable physical properties of the drug has engendered a variety of approaches in innovative formulations which have had significant impact on the

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pharmacokinetics, tissue distribution, and efficacy/toxicity ratios. An excellent review has recently been published.[11]

It is believed that the attenuated nephrotoxicity observed with the more recent liposomal formulations is at least, in part, ascribable to the rapid clearance of the colloidal particles by the reticuloendothelial system, leading to lower C_{max}, higher V_D, and a more sustained release of the drug due to the resultant depot effect;[11] reticuloendothelial sequestration observed with certain formulations perhaps serves as a form of passive drug-targeting and may likely be of benefit in conditions such as visceral leishmaniasis.[17;18] However, early reports in the literature indicate that highly dispersed, monomeric AmB enhances its selectivity while decreasing its toxicity,[19–21] suggesting that toxicity may be related to the physical state of the drug.[22] These earlier studies were performed with surfactants, some of which unfortunately showed synergistic toxicity with AmB,[19;21] and it was desirable for purposes of testing the hypothesis that antifungal and toxic properties of AmB are dissociable with derivatives of AmB that would be intrinsically water-soluble without the need of any excipients.

Inspired by some of the AmB derivatives obtained by semi-synthesis by Carreira's research group,[23–25] and given that our laboratory has considerable expertise with polyamine chemistry, we sought to couple spermine to the carboxylic acid group of AmB using standard carbodiimide chemistry. The solubility of AmB proved to be a significant impediment, and the only solvent that was amenable was dimethylformamide (DMF), a polar, high-dielectric solvent which, coupled with the accidental omission of scavenging agents, favored a serendipitous and unexpected side-reaction to preponderate, yielding a fully water-soluble (> 45 mg/mL) mixture of *N*-acylurea/N-alkylguanidine derivative of AmB. This derivative were found to be monomeric by spectrophotometry, exhibited equipotent antifungal activity against *C. albicans*, and elicited toll-like receptor-2 (TLR2) and TLR4 driven NF-κB responses in reporter gene assays to the same extent as the parent molecule.

Materials and Methods

All of the solvents and reagents used were obtained commercially and used as such unless noted otherwise. Moisture or air sensitive reactions were conducted with anhydrous solvents under argon atmosphere in oven dried (120°C) glass apparatus. Solvents were removed under reduced pressure using standard rotary evaporators. Flash column chromatography was carried out using Silica gel 60 (230–400 mesh), and thin layer chromatography was performed using Silica Gel HLF, pre-coated glass plates. All yields reported refer to isolated material judged to be homogeneous by thin-layer chromatography and NMR spectroscopy. Unless noted otherwise, NMR spectra were recorded with the chemical shifts (d) reported in ppm relative to Me_4Si (for 1H) and $CDCl_3$ (for ^{13}C) as internal standards respectively.

Synthesis of *Bis*-dimethylaminopropylcarbodiimide [N^1-((3-(dimethylamino)propylimino) methylene)-N^3,N^3-dimethylpropane-1,3-diamine] (Scheme 1)

2.5 g of 1,1′-thiocarbonyldiimidazole (**2**, 14 mmol) was heated in 50 mL toluene at 110°C, to which was added 1.8 equivalents (2.6 g, 25 mmol, 3.2 ml, d=0.812) of N^1,N^1-dimethylpropane-1,3-diamine. The reaction mixture was allowed to reflux at 110°C overnight. The thiourea **3** (Scheme 1) was isolated by using a binary DCM/MeOH system on a 40 g normal-phase RediSep Rf silica cartridge using a CombiFlash Companion (Teledyne-Isco, Lincoln, NE) flash chromatography system (Rf: 0.3, 0.927 g, 3.77 mmol, 27% yield). The thiourea **3** was redissolved in DCM and subjected to desulfuration[26] by stirring with 2.0 equiv. of HgO for 2h at room temperature. After filtration of the mercuric oxide through celite, 2.0 mL of HCl in dioxane was added and the solvent evaporated to furnish the

bis(dimethylaminopropyl)carbodiimide **4** (0.4038 g, 1.9 mmol, 50% yield). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.7–1.77 (m, 4H), 2.23 (s, 12H), 2.34 (t, J = 7.12 Hz, 4H), 3.28 (t, J = 6.8 Hz, 4H) ^{13}C NMR ($CDCl_3$, 125 MHz) δ 29.38, 44.82, 45.54, 56.93. MH^+: 213.212.

Synthesis of AmB-Carbodiimide Adducts (Scheme 2)

To a solution of 43.3 mg of AmB (**1**, 46.5 μmol) in 20 ml dimethylformamide (DMF) was added 2.0 equivalents of the carbodiimide **4**, and a catalytic amount of 4-Dimethylaminopyridine (DMAP). The solution was allowed to stir overnight. The solvent was evaporated, and the resultant solid (45.7 mg) was analyzed by LC-MS as described below and found to be a near-equal mixture of the *N*-acylurea/*N*-alkylguanidine di-adduct **5** (48%) and *N*-alkylguanidine mono-adduct **6** (46%), with traces of the *N*-acylurea mono-adduct **7**, the structures of which are shown in Scheme 2. Further purification after redissolving the crude solid in a minimal volume of MeOH, and precipitation by adding it to a large volume of ice-cold diethyl-ether resulted in a substantial enrichment of the *N*-acylurea/*N*-alkylguanidine di-adduct (>85%). The derivative was freely soluble in pure water at 45 mg/ml, the highest concentration tested. This material was used in all further assays.

LC-MS Characterization

The AmB derivative was analyzed by reverse-phase LC-ESI-MS performed on a Shimadzu LC system (LC-10AD binary pumps, SCL-10A diode array detector) using a Zorbax 3.0 mm × 150 mm 3.5 μm stable-bond C_{18} reverse-phase column with a forty-minute binary gradient (CH_3CN/water, 0.1% HCOOH) from 5% to 95% of CH_3CN. ESI-MS data was acquired on an Agilent LC/MSD-TOF instrument with a mass accuracy of 3 ppm and a range of 100 – 3500 Daltons. Calibration drift was minimized on a scan-by-scan basis to less than 2 ppm by using internal standards corresponding to 922.0001 and 2721.0201 marker ions infused concurrently through a second nebulizer in the ionization chamber. MS acquisition was commenced 10 min after injection.

Absorption Spectrophotometry

Absorption spectra of reference AmB in DMSO and the AmB derivative in water were measured using a SpectraMax Plus 384 instrument (Molecular Devices, Sunnyvale, CA) from which appropriate solvent-only blank spectra were subtracted.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of reference AmB (stock solution at 1 mg/mL in DMSO) and the water-soluble AmB adduct (stock solution at 1 mg/mL in H_2O) were determined by broth microdilution method[27] as per NCCLS guidelines. This assay has been described previously in detail for antibacterial compounds.[28] Compounds were two-fold serially diluted in a 384-well microtiter plate in Sabouraud's Dextrose Broth using a Biotek Precision 2000 automated microplate pipetting system. A single colony of *Candida albicans* ATCC 26278 was dispersed in Sabouraud's Dextrose Broth and was added to the wells with appropriate vehicle-only controls. All experiments were done in quadruplicates. The microtiter plates were incubated overnight at 37°C. The plates were read at an absorbance of 600 nm.

TLR2 and TLR4-Specific Reporter Gene assays for Assessing Agonistic Potencies

Human embryonic kidney 293 (HEK-293) cells stably transfected (individually) with either human TLR2, or TLR4 were obtained from InvivoGen, Inc. (San Diego, CA) and were additionally nucleofected with 1 μg of purified *pnifty2* reporter plasmid (InvivoGen) using an Amaxa Nucleofector (Gaithersburg, MD). The *pnifty2* reporter plasmid expresses a

secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB and AP-1 transcription factors. Stable transfectants were selected, expanded, and maintained in HEK-Blue™ Selection Medium (InvivoGen) containing zeocin (10 μg/ml) and normocin (200 μg/ml). The induction of NF-κB was quantitated in a rapid-throughput, homogeneous format as follows: cells were incubated at a density of ~10^6 cells/ml in a volume of 80 μl/well, in 384-well, flat-bottomed, cell culture-treated microtiter plates until confluency was achieved, and subsequently stimulated with graded concentrations of TLR agonists using an automated BioTek Precision 2000 Liquid Handler. SEAP in the medium was assayed spectrophotometrically using an alkaline phosphatase-specific chromogen (present in HEK-detection medium as supplied by the vendor) at 650 nm. These assays have been described in detail previously by us.[29–31]

Results and Discussion

Our original intent, as mentioned earlier, in seeking to attach spermine to the carboxylate of AmB (Fig. 1) in order to test if the polyamine would confer more favorable solubility began earnestly, if somewhat naïvely, with *N*-Boc protection of the mycosamine amine followed by coupling of Tri-Boc spermine[30;32] using standard coupling procedures. Having overcome successive solubility and stability issues, and after purifying in very small yields to homogeneity the final precursor, deprotection of the Boc groups with trifluoroacetic acid resulted, to our dismay, in instantaneous and complete degradation of the molecule. Chastened, we wanted to test if AmB could be coupled at all, even non-regioselectively, to the unprotected polyamine using the more polar 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDCI). Solubility issues left us with no choice but to carry out the reaction in DMF. To our surprise, aqueous workup showed the distinct yellow-orange color of AmB partitioning largely into the water layer. LC-MS analysis of the aqueous layer indicated the presence of multiple isobaric species which eluted off the column at different times, but with masses corresponding to AmB-EDCI adduct which we assigned to the more stable and commonly encountered rearranged *N*-acylurea side-product (Scheme 2).[33–35] We reasoned (incorrectly, as it turns out) that the multiplicity of isobaric species could be a consequence of *N*-acylurea regioisomers (EDCI being non-symmetrical), and hypothesized that a symmetric carbodiimide with two amines would help resolve the isobaric problem whilst affording even greater solubility to the adduct. Hence we synthesized the *bis*(dimethylaminopropyl)carbodiimide **4** (Scheme 1). Reaction of AmB with **4**, to our surprise, yielded an unexpected *bis*-adduct in abundance, in addition to two isobaric products with substantially different retention times (Figs. 2 and 3, Peak 1). Closer examination of mass chromatograms acquired under different fragmenter voltages clearly showed that only one species ionized as both proton and sodium adducts (Fig. 3, Peak 2), and also showed classic -$OH^{\bullet}$ fragments, indicating the presence of a free -COOH group, and thereby implicating the amine as the alternate nucleophile. Although we had not anticipated finding an *N*-alkylguanidine adduct, reactions of amines and carbodiimides to furnish substituted guanidines have indeed been reported in the literature.[36;37] The other mono-adduct corresponding to the *N*-acylurea adduct ionized solely as singly charged proton adduct at higher fragmenter voltages (Fig. 3, Peak 3) and as $+2H^+$ doubly charged species at lower voltages (data not shown). The use of two equivalents of the carbodiimide was fortuitous in retrospect, for it provided an insight into the relative reactivity of the amine and the carboxylate of AmB with the carbodiimide that we perhaps would not have gained had we used an excess of the reagent and obtained entirely the di-adduct **5**.

Precipitation of the crude product in diethyl ether results in substantial enrichment (>85%) of the *N*-alkylguanidine/*N*-acylurea di-adduct **5**, which dissolved freely in water to more than 45 mg/mL (the highest we could test), which is >45,000 the aqueous solubility of the native polyene macrolide. The absorption spectrum of **5** in water (Fig. 4) indicates that the

predominant form is the monomeric one.[38;39] Gratifyingly, the adduction of the alkylaminocarbodimides to both the amine and carboxylic acid groups of AmB does not perturb significantly the function of the molecule, and the anti-fungal activities of reference AmB and **5** against *C. albicans* are quite comparable (Fig. 5). The availability of structurally well-defined, and intrinsically highly water-soluble AmB derivatives such as **5** should allow for a critical reexamination of such concepts as aggregation-dependent selectivity and toxicity given that the critical micellar concentrations of **5** are expected to be orders of magnitude higher than AmB.[40–43]

In addition to the dose-limiting and occasionally fatal nephrotoxicity of AmB, acute infusion-related febrile reactions occur frequently. These are thought to be related to proinflammatory mediator production as a consequence of the activation of toll-like receptors (TLRs), especially TLR2 and TLR4.[44–47] Reporter gene assays for both these TLRs are in routine use in our laboratory,[29] and it was of interest to compare TLR2 and TLR4 agonistic potencies of **5** relative to AmB. As shown in Fig. 5, 5 is more potent than AmB in activating TLR2, while the water-soluble adduct is significantly less active in engaging TLR4 (Fig. 5, bottom panel) which would suggest that the adduct **5** may be less proinflammatory. This is currently being tested using *ex vivo* human blood model systems employing assays of proinflammatory cytokine release and indices of monocyte and natural killer lymphocytic activation, and will be the subject of a future publication.

The assumption concerning the origin of nephrotoxicity has been one of dose-dependent direct cytotoxicity on renal epithelium. This notion is partly supported by observations that cumulative plasma concentrations (and therefore possibly renal exposure to the drug) is lower with the less toxic liposomal preparations (such as AmBiosome and Abelset), compared to Fungizone.[11;48–51] An alternate, hitherto unexamined, and testable hypothesis is that given the substantial TLR-driven proinflammatory activity of AmB (or of its more water-soluble metabolites), mesangial inflammation[52–55] in the renal parenchymal interstitium, rather than tubular damage is the primary cause of nephrotoxicity. If this hypothesis is correct, then a bolus dose of **5** should result in augmented nephrotoxicity, in which case the rational approach to limiting AmB toxicity would be in abrogating its TLR stimulatory activity. All known TLR2 and TLR4 agonists are amphipathic molecules as is AmB,[29] and TLRs, unlike classic receptors, are pattern-recognition molecules. Reducing amphipathicity by masking the polyhydroxyl domain may be one approach. These aspects are being examined.

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Abbreviations

AmB Amphotericin B

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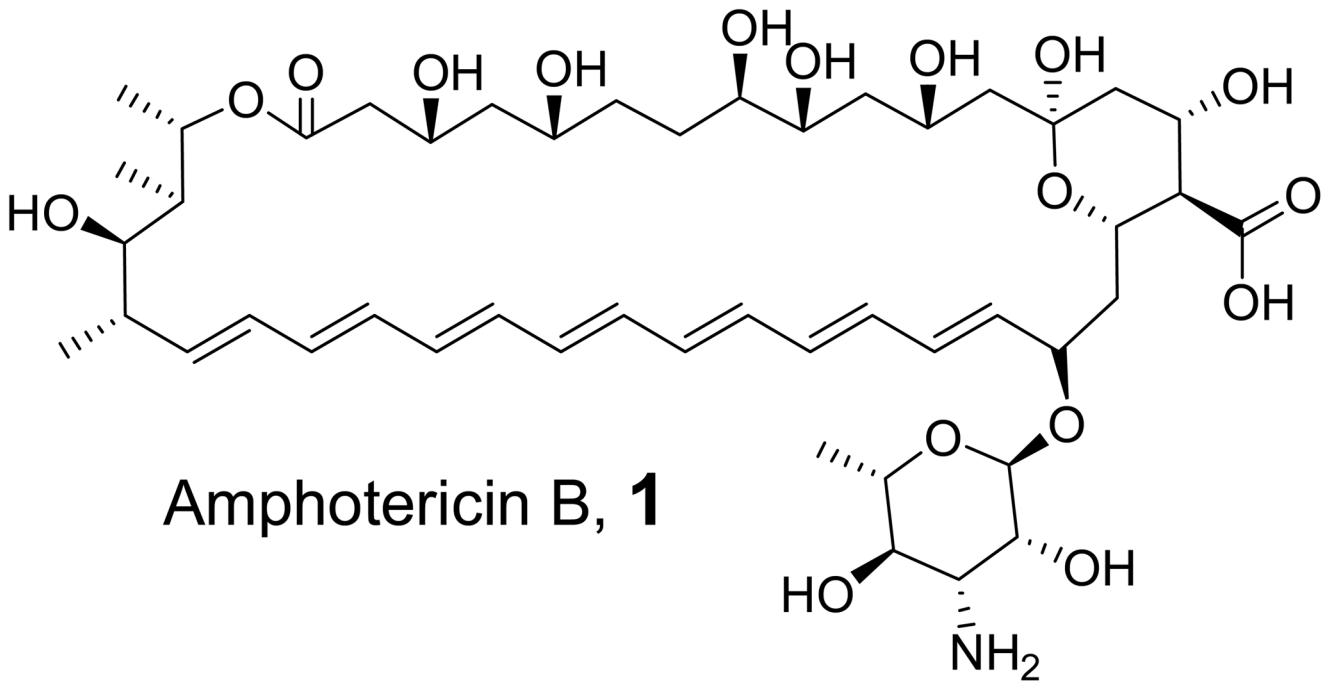


Fig. 1.
The structure of Amphotericin B.

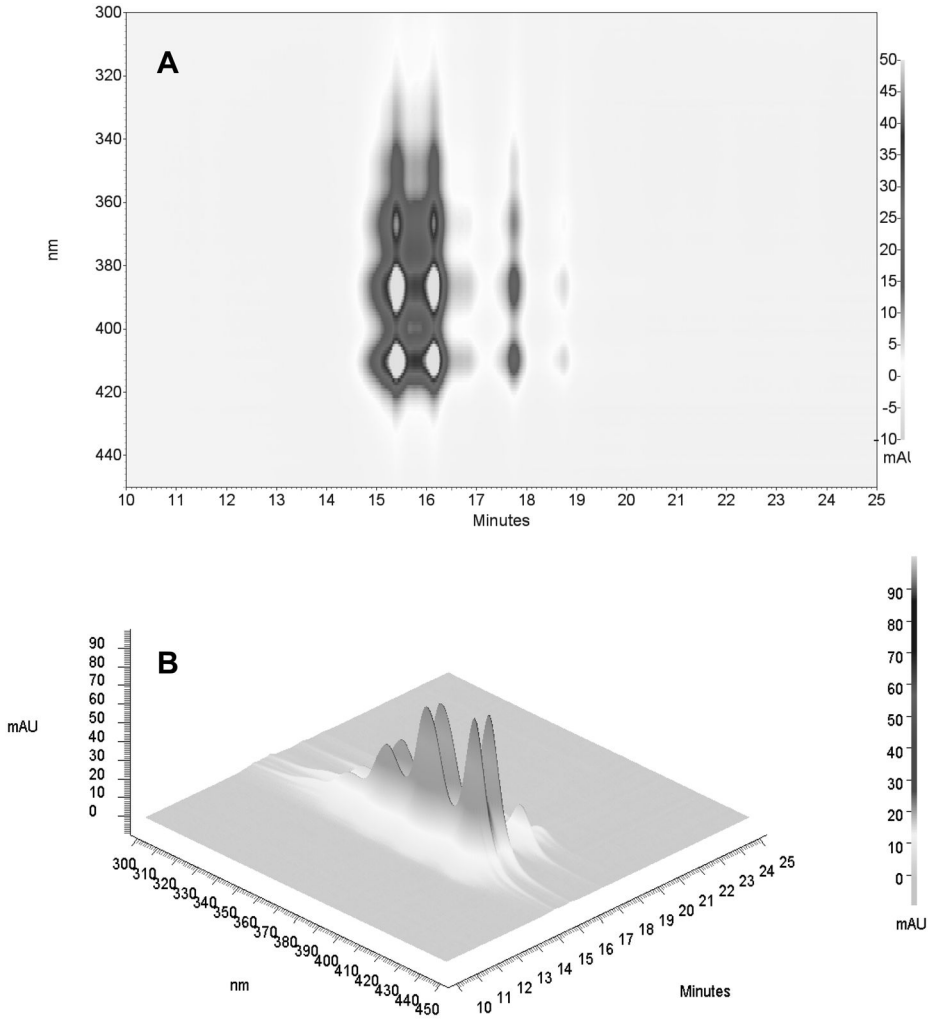


Fig. 2.

2D (A) and 3D (B) plots of absorbance-versus-time profiles from photodiode array detector output of a chromatographic separation of the crude AmB-DMAPCI adducts. All three major bands show absorption spectra characteristic of AmB. The corresponding mass spectra are shown in Fig. 3.

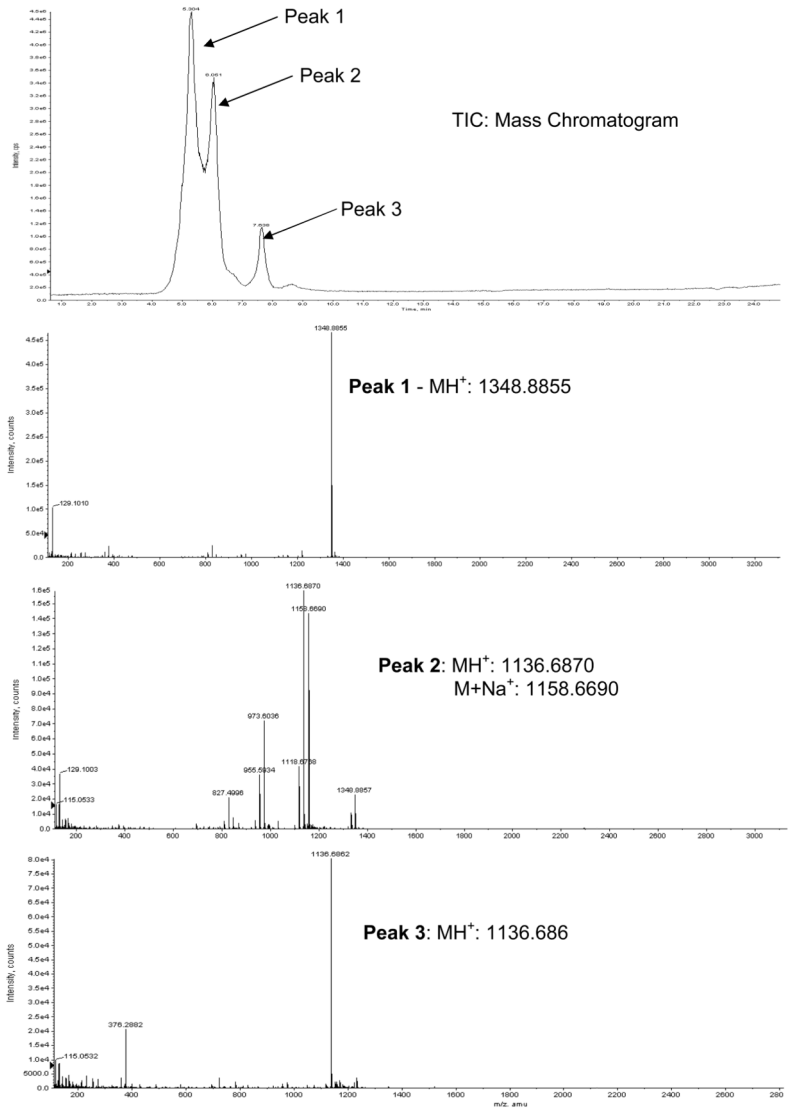


Fig. 3.

Positive ion ESI MS spectra of the chromatographic separation of the crude AmB-DMAPCI adducts. Data were acquired on an LC/MSD-TOF with a mass accuracy of 3 ppm. Note that Peaks 2 and 3 are isobaric; Peak 2, has a free carboxylate that ionizes also as the sodium adduct. The 1118.6788 amu signal in Peak 2 is also diagnostic of a carboxylate (-$OH^{\bullet}$ fragment; M-17.0027).

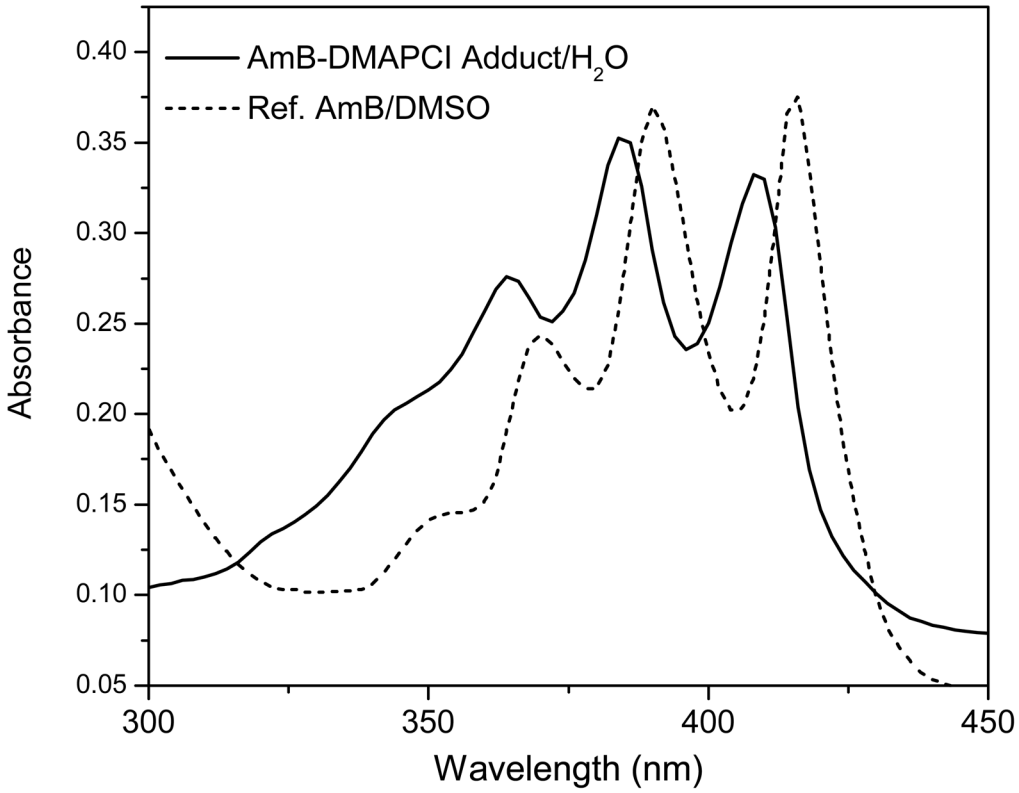


Fig. 4.
Absorption spectra of reference AmB (in DMSO) and the AmB-DMAPCI derivative (in water).

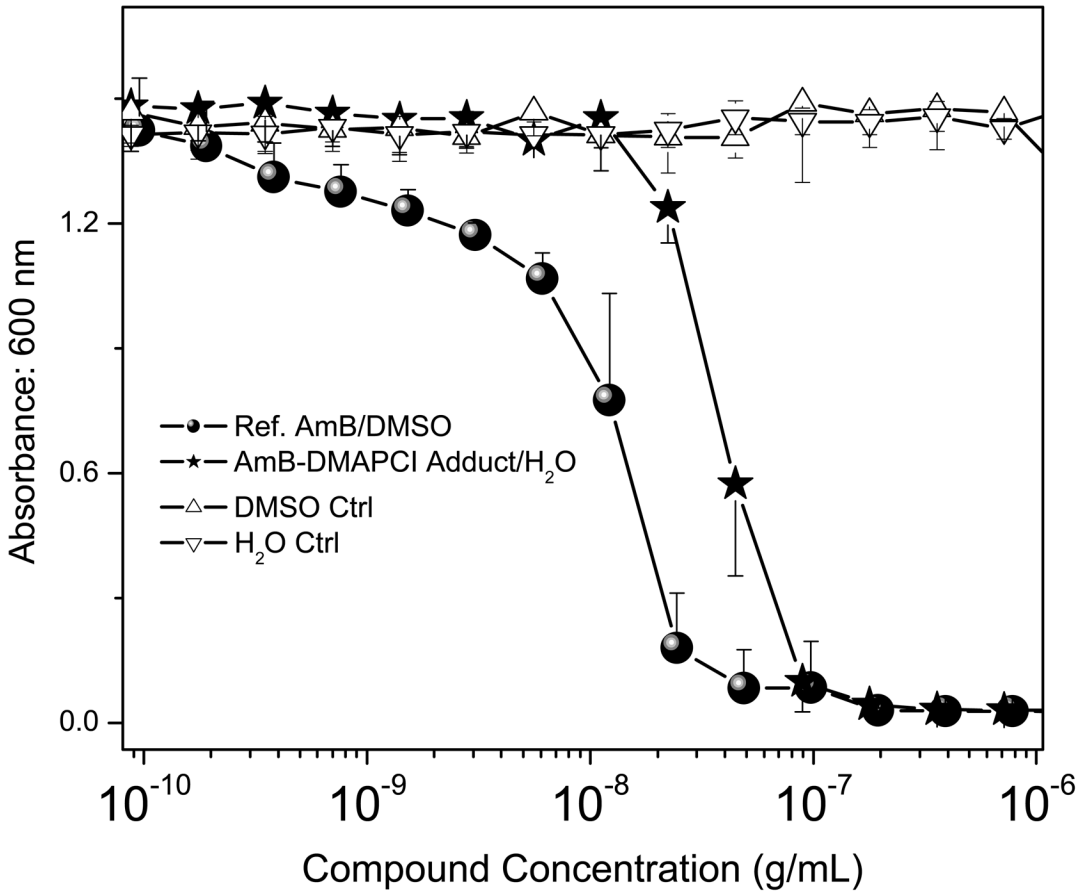


Fig. 5.

Inhibition of *C. albicans* growth by reference AmB (in DMSO) and the AmB-DMAPCI derivative (in water) determined by microplate dilution method. Data represent means and SD obtained on quadruplicates.

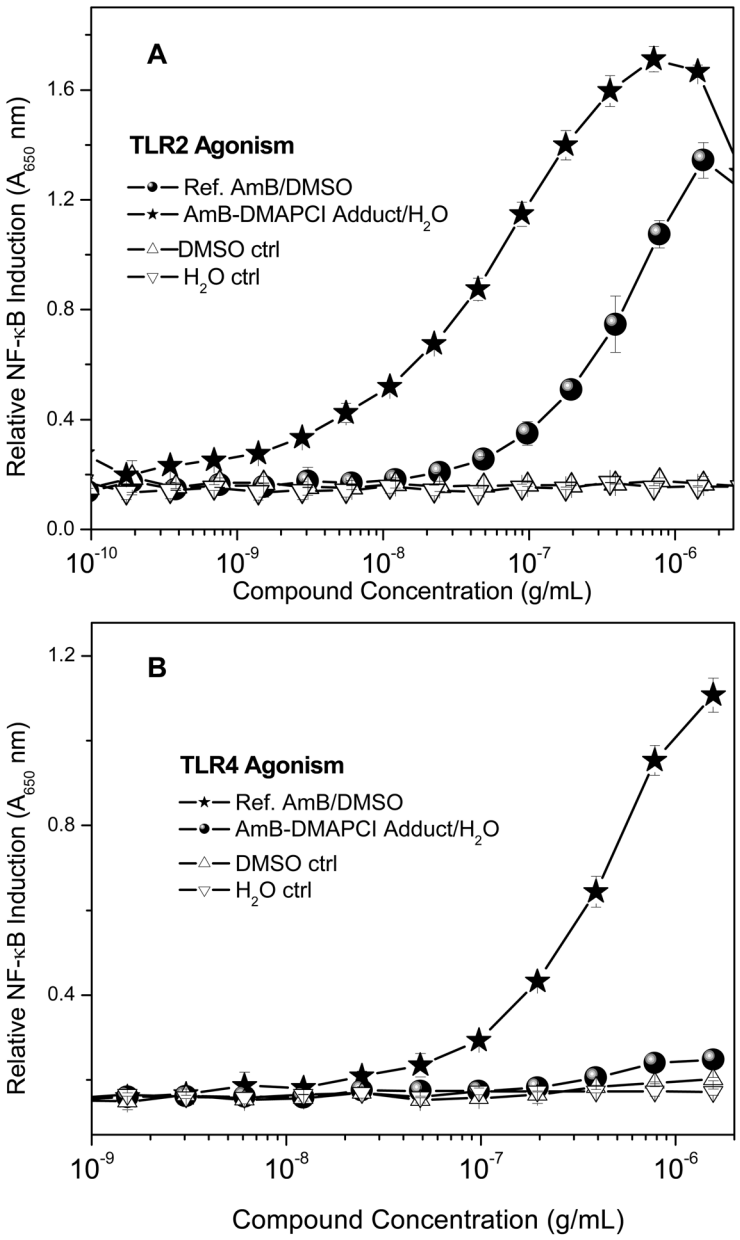
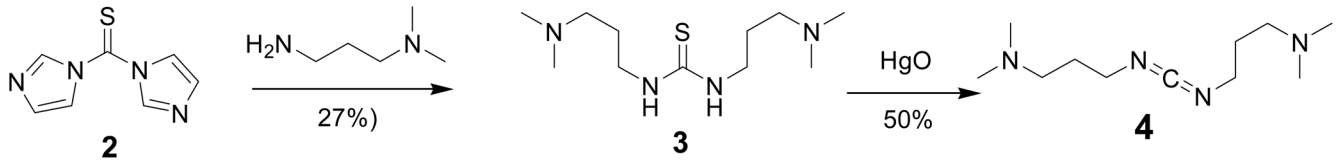
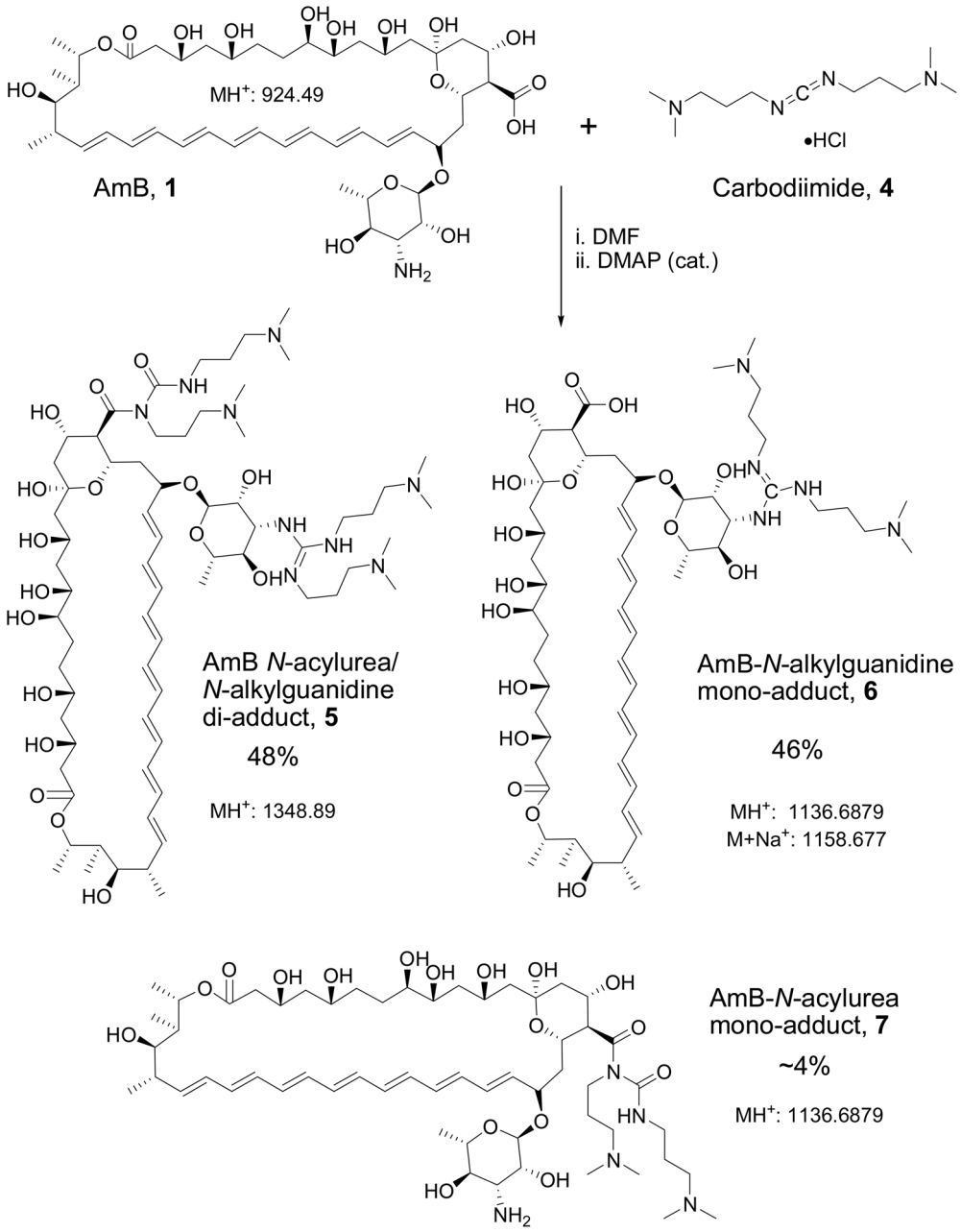


Fig. 6.

TLR2- (A) and TLR4- (B) dependent activation of NF-κB by reference AmB (in DMSO) and the AmB-DMAPCI derivative (in water) determined using TLR-specific reporter gene assays. Data represent means and SD obtained on quadruplicates.



Scheme 1.
Synthesis of *bis*(dimethlaminopropyl)carbodiimide



Scheme 2.
Synthesis of AmB-DMAPCI adducts.