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Article

Amphotericin B Tamed by Salicylic Acid

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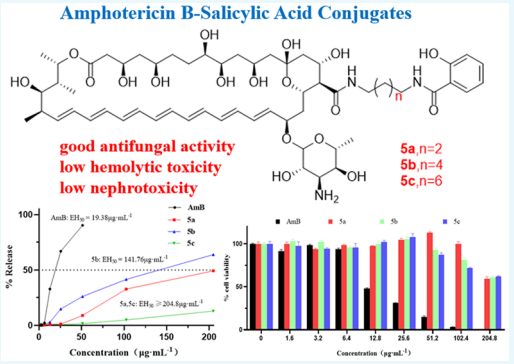
ABSTRACT: Although Amphotericin B (AmB) is considered as the "gold standard" treatment for deep fungal infections, owing to its excellent antifungal effect, it often causes severe hemolytic toxicity and nephrotoxicity, which limits its clinical use. We designed and synthesized AmB derivatives by attaching salicylic acid (SA) to the carboxyl group and confirmed their structures using ^{1}H NMR, ^{13}C NMR, HR-MS, and IR. We evaluated its biological activity *in vitro* and measured its ultraviolet−visible (UV−vis) absorption spectrum. The AmB-SA conjugates exhibited good antifungal effects against *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans* compared with AmB, and the renal cytotoxicity toward HEK 293T cells *in vitro* was significantly reduced, with almost no nephrotoxicity in the therapeutic window of the drug. At the same time, the hemolytic toxicity was significantly reduced. Therefore, modification of AmB by introducing SA is an effective strategy to maintain the broad antifungal activity of AmB and reduce its cytotoxicity. These AmB derivatives could be applied in clinical therapy in the future.



1. INTRODUCTION

Fungal infections are one of the most difficult diseases to manage in humans. Approximately 1.5−2 million people die from fungal infections each year, surpassing malaria or tuberculosis.[1,2] The COVID-19 outbreak has sweep the world and simultaneously increased the risk of fungal infections; thus, the treatment of fungal infections has become a formidable challenge.[3] Therefore, highly effective antifungal drugs with lesser toxicity need to be developed urgently. Although new antifungal drugs such as triazoles and echinocandin have been discovered, amphotericin B (AmB) is still the most widely used systemic antifungal agent.[4,5] AmB, a class of polyene macrolides isolated from the culture of *Streptomyces*, has been the "gold standard" antifungal drug since 1960.[6,7] The mechanism of action of AmB remains controversial, and the most accepted is that AmB preferentially binds with ergosterol and forms trans-membrane channels, which causes the leakage of intracellular K^+ and Mg^{2+} ions and loss of intracellular nutrients leading to cell death.[8−10]

AmB is a double-edged sword that exhibits excellent therapeutic effect, wide antifungal spectrum, and low drug resistance; however, it causes high toxicity to mammalian cells, such as nephrotoxicity, hepatotoxicity, and hemolytic toxicity.[11] Therefore, the need for AmB derivatives, which exhibit high antifungal activity and low or no-toxicity, is urgent. In the past decades, carboxylic acid-attached,[12−14] mycosamine-attached,[15,16] and double modified AmB derivatives[17,18] with diverse functional groups have been developed.[19] Carreira et al. synthesized AmB derivatives through double reductive alkylation of mycosamine, whose *in vitro* antifungal activity was 15 times that of AmB.[20] Similarly, Regen et al. synthesized a series of molecular umbrella-AmB conjugates with good antifungal activity and low hemolytic toxicity and nephrotoxicity.[21,22] Several other chemists and pharmacists have also been devoted to the modification of AmB and have obtained inspiring results. However, AmB derivatives for clinical use still could not be identified. This encouraged us to synthesize novel AmB derivatives with low or no toxicity to mammalian cells and high antifungal activity.

Salicylic acid (SA) is naturally found in willow bark, white pearl leaves, and sweet birch trees. It is an important pharmaceutical compound whose medicinal value has been studied for a long time. SA derivatives also show good biological activity. For example, aspirin, a well-known SA derivative, has analgesic, antipyretic,[23] anti-inflammatory,[24] and anticancer effects,[25] and it also inhibits platelet aggregation and prevents embolism.[26] Zhou et al. explored the synergistic effect of aspirin and AmB, and the *in vitro* study results showed that the antifungal effect of AmB was significantly enhanced when combined with aspirin.[27] Ngaini et al. obtained a series of compounds with good antifungal effects by introducing thiourea groups. They found that the presence of hydroxyl, carboxyl, and halogen groups played an important role in

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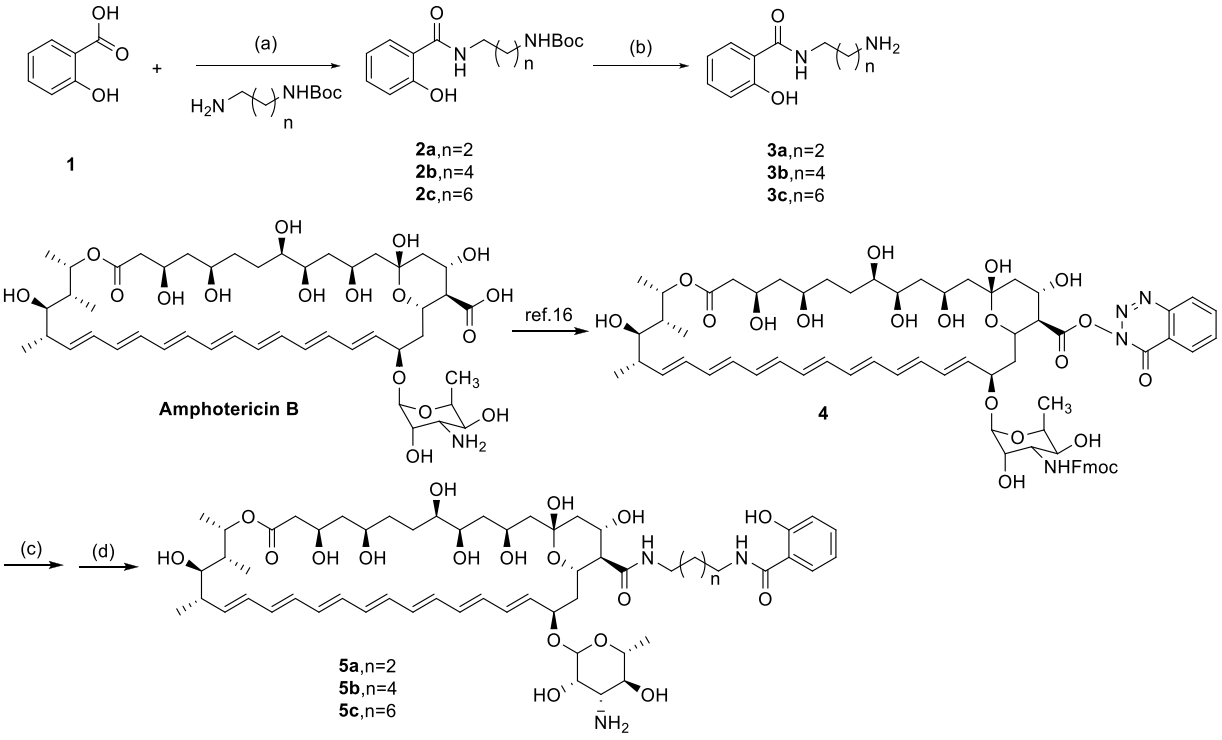
Scheme 1. Synthesis of Target Compounds 5a−5c[a]



[a]Reagents and conditions: (a) Dry tetrahydrofuran (THF) and dicyclohexyl carbodiimide (DCC), stirred at 0°C for 30 min, then warmed to room temperature for 18 h; (b) Dichloromethane (DCM) and trifluoroacetic acid (TFA), 30 min, room temperature; (c) *N,N*-dimethylformamide (DMF) and diisopropylethylamine (DIPEA), in the dark at room temperature for 30 min; (d) DMF and piperidine, in the dark at room temperature for 30 min.

improving the antifungal activity of the compounds.[28,29] SA is also a lipid-soluble organic acid, and studies have shown that the lipophilicity of aromatic rings contributes to the improvement of its biological activity.[30] Additionally, we can say that SA derivatives exhibit good biological activity on the basis of the concept that aspirin has a synergistic effect with AmB. This inspired us to design and synthesize AmB derivatives that covalently linked SA and AmB with different alkyl chains. As expected, the cytotoxicity of the AmB-SA conjugates was significantly reduced, and the antifungal activity was comparable to that of AmB.

2. RESULTS AND DISCUSSION

2.1. Chemistry. The synthesis of **5a−5c** compounds is shown in Scheme 1. First, the reaction of SA with commercially available *N*-Boc-amine (1.1 equiv) in dry THF resulted in the corresponding adducts **2a−2c**; hydrolysis of the labile Boc protection group by TFA led to free amines **3a−3c** in 84%, 87%, and 89% yields, respectively. Then, **3a−3c** free amines were used to modify the activated carboxylic group of AmB. Finally, the labile Fmoc protection group was removed by the action of piperidine, leading to the free amino AmB-SA derivatives **5a−5c** in 40%, 44%, and 48% yields, respectively.

Their structures were confirmed using ^{1}H NMR, ^{13}C NMR spectroscopy, IR, and HR-MS. The introduction of SA into AmB caused a downfield shift of signals (δ = 7.82, 7.38, and 6.9 ppm) in the ^{1}H NMR spectra of compound **5a**. However, when the four hydrogens of the alkyl chain were introduced, the signals shifted highfield (δ = 3.92−4.19 ppm) in the ^{1}H NMR spectra, and all characteristic hydrogen signals of AmB were also in accordance with the literature.[21,22] The positively charged molecular peak $[M + H]^{+}$ was measured in the mass spectra of **5a**, and HR-MS analysis also confirmed the structure of **5a**. However, given the complexity of the compound, we also used ^{13}C NMR and IR to confirm its structure. In the ^{13}C NMR spectrum, there were three signals at 177.83, 176.43, and 174.21 ppm corresponding to the three carbonyl carbons. There was a signal of the carbonyl group of the lactone ring at δ = 176.43, indicating that the large lactone ring is not opened. Four signals at 22.34, 21.22, 20.46, and 15.73 ppm corresponded to the four methyl groups of the parent AmB. After the introduction of SA into AmB, six benzene carbon signals in SA were observed at 164.64, 133.29, 131.59, 122.61, 121.88, and 119.19 ppm, respectively. This indicated that the SA fragment was successfully attached. The IR spectra of **5a** contained the absorption bands at 1640 cm^{-1} (characteristic of the −CONH− group) and 1450 cm^{-1} (characteristic of the benzene ring structure of SA). These results indicate that the structure of the AmB-SA derivatives is correct. ^{1}H NMR, ^{13}C NMR, IR, and HR-MS data for **5a−5c** are provided in the Supporting Information. ^{13}C NMR spectra contain some minor impurities. We think that it could be residual solvents and some small impurities. We think that there may be residual solvents of DMF (δ = 167.12, 35.04, 35.32) and methyl *tert*-butyl ether (δ = 49.04, 80.59, 26.88, 27.59, 27.79). We also performed HPLC analysis (Column, Symmetry C18 4.6 mm × 250 mm; mobile phase, (A) 0.6% acetic acid in water and (B) methyl alcohol; flow, 1 mL/min and λ = 409 nm). HPLC was applied to determine whether there are rotamers of the amides. The results showed that compounds **5a−5c** are of high purity and there are no rotamers of the amides. It could be seen from HPLC that the retention times of **5a−5c** were relatively close.

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(Figures S17−S19) According to the United States Pharmacopoeia, the purity reference standard for Amphotericin B is 88.91%, because Amphotericin B contains the impurities Amphotericin A and Amphotericin C and these impurities are difficult to remove completely. So, the derivatives inherit the impurities. According to relevant literature reports, the purity of its derivatives is higher than that of Sigma Amphotericin B.[14] Therefore, the conclusion that the structure of modified AmB-SA derivatives is reliable.

2.2. *In Vitro* Antifungal Assays. Conjugates **5a−5c** were screened for their *in vitro* antifungal activity against *C. albicans*, *C. glabrata*, and *C. neoformans* to determine the minimum inhibitory concentrations (MICs) of each antifungal agent. The concentration of fungi were adjusted to 2000 CFU·mL^{-1} with RPMI 1640 medium. Solutions of **5a−5c** were prepared at 1.6 mg·mL^{-1} using DMSO. Serial dilutions of the test compounds **5a−5c** were prepared to final concentrations of 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg·mL^{-1}. The MIC was defined as the lowest drug concentration that resulted in complete inhibition of visible growth. *C. albicans* and *C. glabrata* were incubated for 24 h at 35 °C, and *C. neoformans* was incubated for 72 h at 35 °C (Table 1 presents the results). The antifungal activity of the three conjugated compounds **5a−5c** was reduced to some extent, at 2−8-fold less than that of AmB. From these results, we concluded that compound **5a**, which had the shortest carbon chain length, had broad-spectrum antifungal activity against all three fungi compared with that of AmB. Table 2 shows the values of AmB, **5a**, **5b**, and **5c** for antifungal potency and toxicity, as well as the comparison with other derivatives in the literature. AmB is a therapeutic compound that is highly toxic to mammalian erythrocytes. Previously reported compounds, such as those lacking the hydroxyl group in C2′,[32] are relatively effective against fungi and have less toxicity; however, the limited synthetic pathways for these derivatives have hindered their further development. The two modified compounds **33** and **34**[10,15,20] had better antifungal activity but were more toxic to mammalian erythrocytes. In contrast, according to our results, the activity of the derivatives decreased to a certain extent, but their toxicity was significantly reduced. It is important to note that the derivatives maintained good and broad antifungal activity.

Table 1. Antifungal Activities of Target Compounds 5a−5c[a]

	MIC/μg·mL^{-1}		
compounds	*C. albicans*	*C. glabrata*	*C. neoformans*
AmB	0.5−1	0.5−1	0.25−0.5
5a	2−4	1−2	1−2
5b	4−8	2−4	2−4
5c	4−8	2−4	2−4

[a]MIC: Minimal inhibitory concentration values are the lowest concentrations required to completely inhibit fungal growth.

2.3. *In Vitro* Hemolysis Tests. The hemolytic toxicities of these derivatives were also investigated. Figure 1 shows the plot of the percentage of released hemoglobin from 4% sheep red blood cells as a function of the concentrations of AmB, **5a**, **5b**, and **5c**. The results showed that AmB began to exhibit hemolytic toxicity at 6.4 μg·mL^{-1} and EH_{50} was 19.38 μg·mL^{-1}. The corresponding EH_{50} value of **5b** was 141.76 μg·mL^{-1} whereas the values for **5a** and **5c** were greater than 204.8 μg·mL^{-1}. The hemolytic toxicity of all three compounds was lower than that of the reference AmB at all concentrations. At a concentration of 51.2 μg·mL^{-1}, the hemolysis rate of AmB was 90% while that of **5c** was 1.6%. Additionally at a concentration of 102.4 μg·mL^{-1}, the hemolysis rate of AmB was 98%, while that of **5c** was only 4.9%. The hemolytic toxicity (EH_{50}) of **5a** and **5c** was 1.4 times lower than that of **5b** and 10.5 times lower than that of AmB. In particular, the hemolytic toxicity of **5c** was almost negligible even when the concentration was as high as 204.8 μg·mL^{-1}. Thus, the hemolytic toxicity of conjugates **5a−5c** was dramatically reduced. These results indicate that the introduction of the SA group is an effective way to obtain AmB derivatives with low hemolytic toxicity.

2.4. *In Vitro* Nephrotoxicity Studies. HEK 293T cells were used to determine the *in vitro* toxicity of the AmB derivatives in mammalian cells (Figure 2). AmB showed obvious toxicity in HEK 293T cells. It can be observed that, when the concentration was at 25.6 μg·mL^{-1}, AmB killed more than 20% of the cells; and 90% of the cells were killed at a concentration of 102.4 μg·mL^{-1}. None of the derivatives **5a**, **5b**, or **5c** showed nephrotoxicity at a concentration of 25.6 μg·mL^{-1}. At a concentration of 51.2 μg·mL^{-1}, **5a** was still not toxic to HEK 293T cells, whereas **5b** and **5c** showed slight nephrotoxicity. Even at a higher concentration of 204.8 μg·mL^{-1}, cell viability was over 50% after treatment with all three derivatives. Thus, none of the AmB-SA conjugates exhibited nephrotoxicity in the therapeutic window; SA is an effective functional group for the modification of AmB.

2.5. Spectroscopic Properties and Self-Association. AmB aggregation is considered the underlying cause of serious toxic side effects in the treatment of deep fungal infections. It has been demonstrated that AmB in aqueous solutions forms macromolecular aggregates, which increases its toxicity.[31] Here, we tested the UV−vis absorption spectra of AmB and its derivatives, and the results are shown in Figure 3. All four characteristic UV−vis absorption peaks of AmB at 407, 385, 365, and 345 nm were retained, and AmB existed as a monomer in methanol. The absorption spectra of **5a**, **5b**, and **5c** in methanol were similar to those of the AmB monomer (Figure 3a). The ratio of the absorption bands is not strongly correlated with the concentration, and the intensity of the absorption bands conforms to the Lambert−Beer law. AmB-SA conjugates and AmB existed in more than two forms in phosphate-buffered saline (PBS) than in methanol solution, with the peak of aggregation at 345 nm and monomer at 407 nm (Figure 3b,c). The ratio of absorbance at 345 and 407 nm (A_{345}/A_{407}) was used to estimate the degree of self-association. A peak I−IV ratio >2 is regarded as aggregation.[31] In PBS, the aggregation degree of AmB-SA also increased with increased concentration, but it was lower than that of AmB. At a concentration of 51.2 μg·mL^{-1}, the aggregation degree values of AmB, **5a**, **5b**, and **5c** were 3.382, 2.518, 2.223, and 2.27, respectively, which were all greater than 2. At this time, the hemolytic toxicity of AmB was significantly increased. In contrast, although the aggregation degree value of AmB-SA was greater than 2, it still exhibited low hemolytic toxicity. Parts b and c of Figure 3 show the absorption spectra of the drug at low and high concentrations in PBS. At a low concentration (6.4 μg·mL^{-1}), the ratios of absorbance were as follows: AmB, 1.62; **5a**, 1.26; **5b**, 1.27; **5c**:, 1.60. Whereas at a high concentration (102.4 μg·mL^{-1}), the ratios of absorbance were as follows: AmB, 3.28; **5a**, 2.47; **5b**, 2.17; and **5c**, 2.21. The absorption spectra of AmB, **5a**, **5b**, and **5c** at the concentrations of 12.8, 25.6, and 51.2 μg·mL^{-1} are shown in Table 3. The results showed that **5a−5c** had a similar degree of

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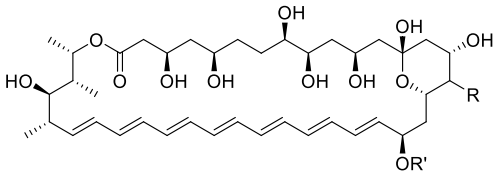
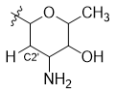
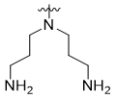
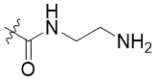
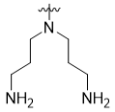
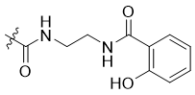
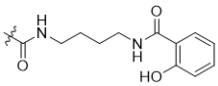
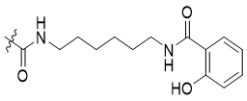
Table 2. Comparison of the Activity of AmB and its Derivatives on Fungal and Mammalian Cells



Compounds	R	R'	MIC in *C. albicans* ($\mu g \cdot mL^{-1}$)	EH_{50}-Erythrocytes ($\mu g \cdot mL^{-1}$)
AmB		Mycosamine	0.5-1	19.38
C2'deOAmB			1	>500
33			0.25	50
34			1	30
5a		Mycosamine	2-4	204.8
5b		Mycosamine	4-8	141.76
5c		Mycosamine	4-8	>204.8

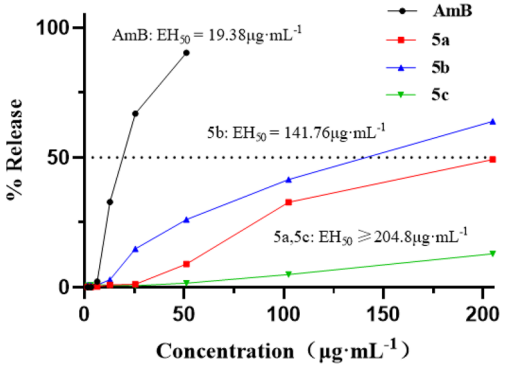


Figure 1. Plot of released hemoglobin (% release) from 4% sheep red blood cells as a function of concentrations of AmB, **5a**, **5b**, and **5c** at 37 °C in phosphate-buffered saline (PBS) and pH 7.4. The optical density of each sample was measured at 540 nm.

aggregation as AmB, and the degree of aggregation increased with increasing concentration. Comparing the antifungal activity and cytotoxicity, we found that the cytotoxicity of AmB-SA conjugates was different from that of AmB. The toxicity of AmB was positively correlated with concentration and the aggregation form was highly toxic to mammalian cells, but AmB-SA still showed very low cytotoxicity.

3. CONCLUSION

Novel AmB derivatives attached with SA were synthesized in this study, their structures and purity are reliable, confirmed by ^{1}H NMR, ^{13}C NMR, HR-MS, IR, and HPLC. All three AmB-SA derivatives showed good antifungal activity against *C. albicans*, *C. glabrata*, and *C. neoformans*. The length of linkage chains has an obvious effect on the antifungal activity of the derivatives. The shortest two-methylene chain derivative, **5a**, exhibited the best antifungal activity. However, the antifungal activity of the derivatives decreased slightly with increasing chain length, whereas both **5b** and **5c** also retained good antifungal activity. The hemolytic toxicity and nephrotoxicity of AmB-SA derivatives were significantly reduced compared to those of AmB. Conjugates **5a**, **5b**, and **5c** showed very low nephrotoxicity, even at high concentrations; **5c** exhibited the lowest hemolytic toxicity and **5b** had higher toxicity than the other two compounds, but still far lower than AmB. Moreover, the hemolytic toxicity of **5a** was between those of **5b** and **5c**. We found that the concentration of aggregation decreased for all three AmB-SA derivatives, and the cytotoxicity of these

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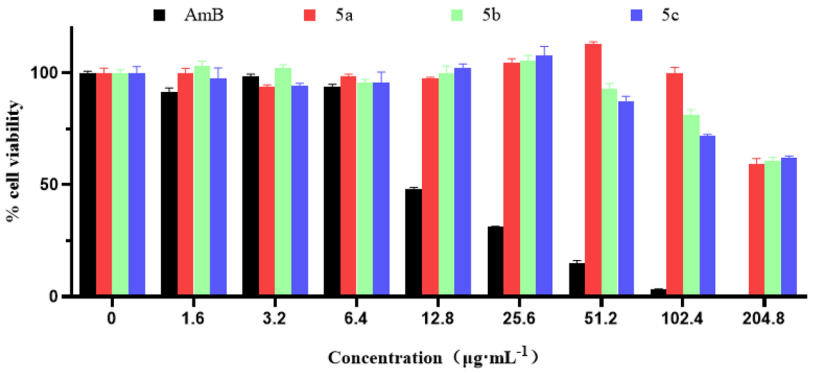

Figure 2. Cell viability of HEK 293T cells treated with **5a**, **5b**, **5c**, and AmB using a cell counting kit-8 (CCK-8 assay). Error bars indicated the mean ± SEM.

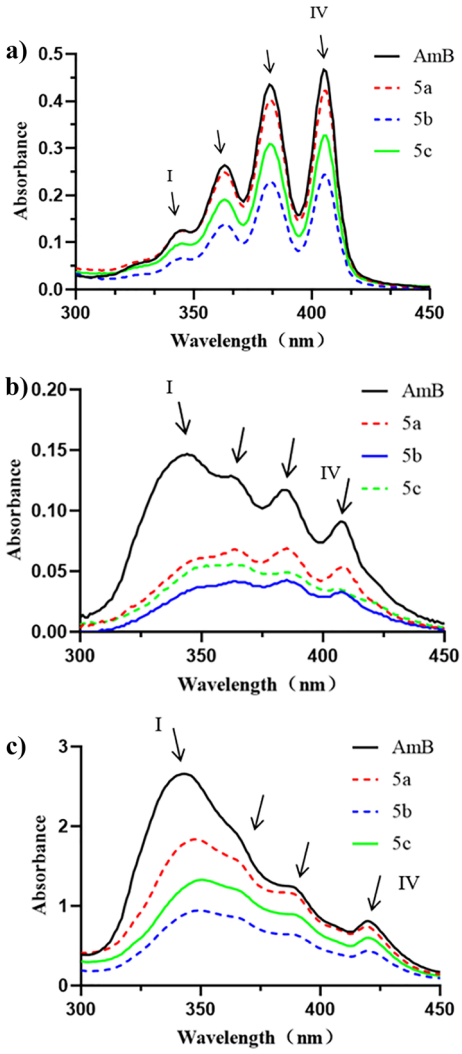

Figure 3. Absorption spectra of AmB, **5a**, **5b**, and **5c** at (a) 6.4 μg·mL^{-1} in methanol, (b) 6.4 μg·mL^{-1} in PBS, and (c) 102.4 μg·mL^{-1} in PBS.

novel conjugates was not correlated with the aggregation state. The promising results encouraged us to put more effort into the evaluation of their *in vivo* safety and antifungal activity. We hope that these derivatives with good antifungal activity and low toxicity could be used in clinical therapy.

Table 3. Absorption Spectra of AmB, 5a, 5b, and 5c at Different Concentrations in PBS

	concentration (μg·mL^{-1})				
compound	6.4	12.8	25.6	51.2	102.4
AmB	1.615	2.267	2.932	3.382	3.279
5a	1.259	1.460	1.919	2.518	2.466
5b	1.273	1.452	1.823	2.223	2.165
5c	1.600	1.770	2.287	2.270	2.210

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c07201.

Discussions of genral information, experimental procedures, and analysis conditions and figures of absorption spectra, NMR and HRMS spectra, IR spectra, and HPLC info (PDF)

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Notes

The authors declare no competing financial interest.

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