European Journal of Medicinal Chemistry 182 (2019) 111639

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Scalable and cost-effective tosylation-mediated synthesis of antifungal and fungal diagnostic 6″-Modified amphiphilic kanamycins

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ARTICLE INFO

Article history:
Received 24 July 2019
Received in revised form
20 August 2019
Accepted 21 August 2019
Available online 22 August 2019

Keywords:
Amphiphilic kanamycin
Antifungal
Fungal infection
Fungal diagnostic
Fluorescent aminoglycoside

ABSTRACT

Amphiphilic kanamycins bearing hydrophobic modifications at the 6″ position have attracted interest due to remarkable antibacterial-to-antifungal switches in bioactivity. In this report, we investigate a hurdle that hinders practical applications of these amphiphilic kanamycins: a cost-effective synthesis that allows the incorporation of various connecting functionalities to which the hydrophobic moieties are connected to the kanamycin core. A cost-effective tosylation enables various modifications at the 6″ position, which is scalable to a 90-g scale. The connecting functionalities, such as amine and thiol, were not the dominant factor for biological activity. Instead, the linear chain length played the decisive role. Amphiphilic kanamycin attached with tetradecyl (C14) or hexadecyl (C16) showed strong antifungal and modest antibacterial activities than with shorter chains (C6–C10). However, increases in chain length were closely correlated with an increase in HeLa cell toxicity. Thus, a compromise between the antimicrobial activities and cytotoxicities, for optimal efficacy of amphiphilic kanamycins may contain chain lengths between C8 and C12. Finally, the described synthetic protocol also allows the preparation of a fluorescent amphiphilic kanamycin selective toward fungi.

1. Introduction

Amphiphilic kanamycin (AK) such as those synthesized from kanamycin A, kanamycin B and tobramycin—all with hydrophobic moieties attached at the 6″-position—has attracted great interest due to their broad spectrum antibacterial and antifungal activities [1–5]. The reported synthetic approaches have allowed studies on structure-activity relationships (SARs), including the effects of connecting functionalities, such as thiol, sulfone, amide, ether, and 1,2,3-triazole) and the optimal chain length of the linear hydrophobic groups. Despite the advances, cost-effective and scalable production of the lead compounds has not been satisfactorily resolved. In addition, there is no AK bearing an amine as the connecting functionality. Amines can amend the solubility or bioavailability of the AK (especially for those with longer (>C14) linear hydrophobic chains) by bringing additional hydrogen bonds or cations. To address these shortcomings, we decided to employ kanamycin sulfate as the starting material and explore toluenesulfonyl as the connecting functionality in the chemical synthesis of AK-carrying amino hydrophobic moieties attached at the 6″-position. Commercially available kanamycin sulfate (containing >95% kanamycin A) is more cost-effective compared to kanamycin B, tobramycin, or other kanamycin-class aminoglycosides (Fig. 1).

2. Rationale, design and synthesis

To implement modifications at the hydroxyl groups of kanamycin, the amino groups need to be protected or masked as azido groups. The former is commonly achieved using carbamate-type protecting groups, e.g. *t*-butoxycarbonyl (Boc) or carboxybenzyl (Cbz) groups. The latter can be done via amine/azide transformation. We favor the use of Boc groups for two reasons. First, the reagent (Boc_2O) is inexpensive and much safer to handle as compared to azide needed for the amine/azide transformation. Second, Boc groups can be removed without trace using TFA, whereas removal of Cbz requires hydrogenolysis using a costly and

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https://doi.org/10.1016/j.ejmech.2019.111639
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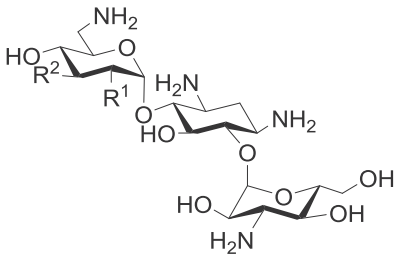


	R^1	R^2
kanamycin A	OH	OH
kanamycin B	NH_2	OH
tobramycin	NH_2	H

Fig. 1. Structure of kanamycin class aminoglycosides.

hazardous metal catalyst. We have previously reported the large scale synthesis of Boc-protected kanamycin (300 g scale) without the need of column purification [6]. The next hurdle is the regioselective incorporation of a hydrophobic group at the 6″-position. This is conducted by selectively converting the 6″-OH into a leaving group, followed by nucleophilic substitution. A bulky 2,4,6-triisopropylbenzenesulfonyl (TIPBS) chloride is commonly used to enable selective incorporation at the 6″-OH [7,8]. However, TIPBSCl is not cost-effective and usually needs to be employed in excess amount (5 equiv.). We believe that toluenesulfonyl (Ts) chloride is a better option. After several attempts, we achieved comparable yields as those obtained using TIPBSCl but with only 2.5 equiv. of TsCl to generate compound **2** (Scheme 1). Furthermore, we demonstrated that the reaction can be conducted at larger scales (ca. 90 g scale). In contrast, the reported synthesis using TIPBSCl was often conducted on a scale less than 5 g. The tosylation site was confirmed from the $^1H-^1H$ COSY of compound **3**.

Following the successful synthesis of tosylated kanamycin derivative, nucleophilic substitution using linear alkylamines (hexyl, octyl, decyl, dodecyl, tetradecyl and hexadecyl amines) followed by column fractionation and deprotection of Boc groups provided the desired products. To compare similar compounds as reported by others, we also used linear alkyl thiols (hexyl, octyl, decyl, dodecyl, tetradecyl, and hexadecyl thiols) as the nucleophiles. Without purification using column chromatography, the Boc groups of the substitution products were removed using TFA and furnished the final AK ready for biological assay. In addition, we synthesized a fluorescent member, **7** using an amide as the connecting functionality. Compound **7** enables fluorescence-based evaluation of microbe selectivity (fungi vs. bacteria), which will be discussed later. Finally, it is important to mention that the tosylated kanamycin derivative can also lead to the synthesis of AK with ether, amide, or sulfone connecting functionalities using established protocols [3,9].

3. Results and discussion

3.1. Antifungal activity of amphiphilic kanamycin

The antifungal activity of newly synthesized AK was tested against a panel of fungi, including *Candida albicans* 64124 (human pathogen, azole-resistant), *C. albicans* MYA2876 (human pathogen, azole susceptible), *C. albicans* B-311 (human pathogen), *C. rugosa* 95−967 (human pathogen), *C. parapsolis* Cas08-0490 (human pathogen, azole-resistant), *C. tropicalis* 95-41 (human pathogen), *Cryptococcus neoformans* H99 (human pathogen), *C. neoformans* VR-54 (human pathogen), *Rhodotorula pilimanae* (ATCC 26423, plant pathogen), and *Fusarium graminearium* B4-5A (plant pathogen), using **K20** as the control. The minimum inhibitory concentrations (MICs) were determined (Table 1). From the MICs, AKs bearing tetradecyl (C14) and hexadecyl (C16) were most potent regardless of the connecting functionalities (amine and thiol). This is consistent with those thiol analogs reported by others. Interestingly, K20 compounds carrying octanesulfonyl groups are only slightly less active than the AK bearing tetradecyl (C14) and hexadecyl (C16) groups, while those with octyl or decyl chains are much less active, especially against *Candida* sp. In addition, strains of the genuses *Cryptococcus, Rhodotorula,* and *Fusarium* become more prone to the increase in chain length, as compounds **4b,c** and **5b,c** attached with octyl or decyl groups started to show increased antifungal activity. Based on these findings, it is concluded that the connecting functionality does not play a key role in level of antifungal activity. Rather, the chain length and the strain of fungi are the main factors that govern the antifungal activity of AK with 6″-modification.

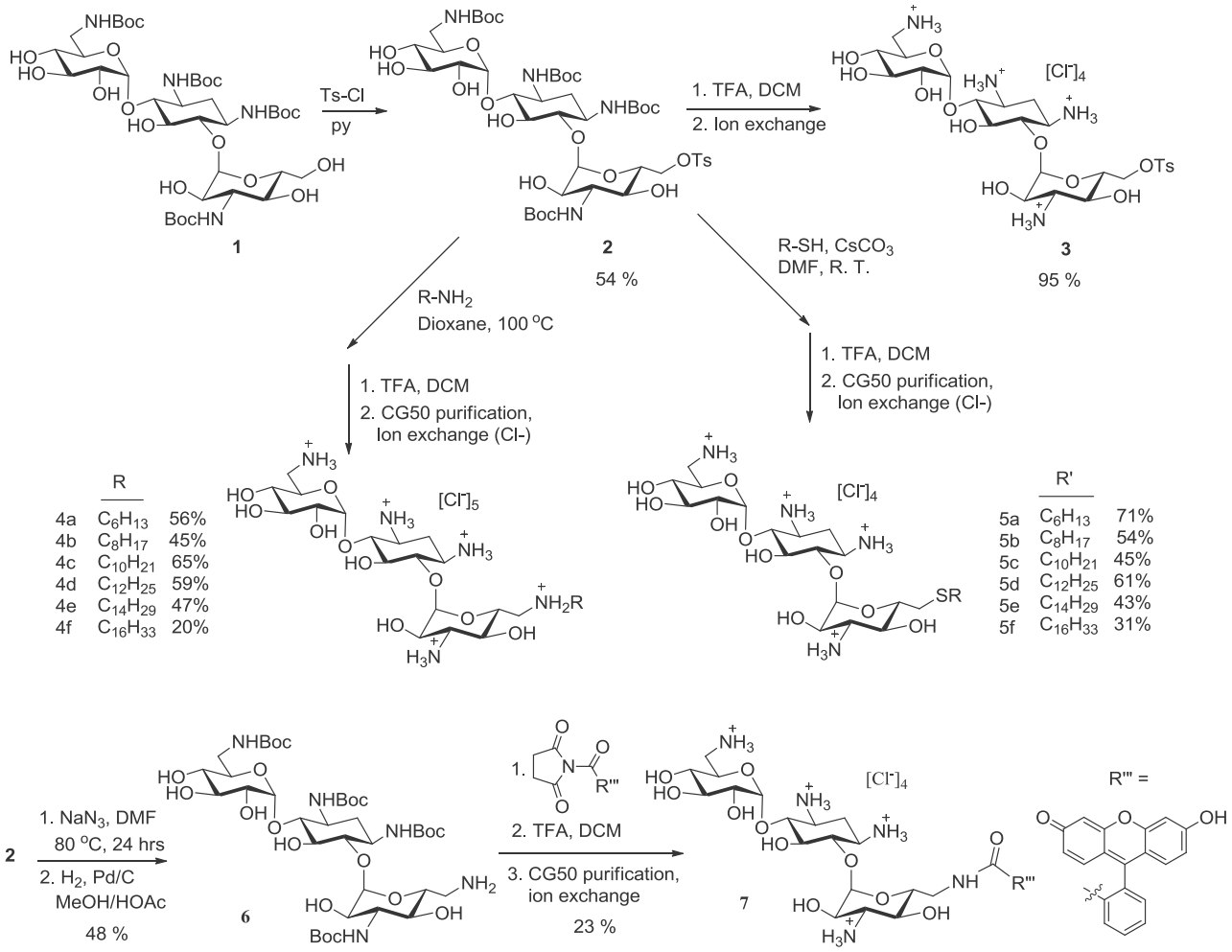
3.2. Antibacterial activity of amphiphilic kanamycin

The antibacterial activity of AK was also tested against a panel of bacteria, including *Escherichia coli* (ATCC 25922, Gram negative, G-), *Staphylococcus aureus* (ATCC 25923, Gram positive, G+), *S. aureus* (ATCC 33591, MRSA), and *S. aureus* (ATCC 43300, MRSA) using kanamycin A and vancomycin as the controls (Table 2). The results showed that these AK exerted only modest antibacterial activity even for those with C14 or C16 chains, and again the connecting functionalities did not have obvious effect. Nevertheless, the antibacterial SAR is similar to the antifungal SAR implying the lack of selectivity between antibacterial and antifungal activity. Interestingly, compound **3** with its toluenesulfonyl group displayed an activity profile like kanamycin A; it was active against aminoglycoside susceptible strains while being inactive against MRSA.

3.3. Cytotoxicity of amphiphilic kanamycin against HeLa cells

Selected AKs with good antifungal activity were tested for their cytotoxicity towards HeLa cells (Fig. 2). The IC_{50} values of the compounds against HeLa cells are presented in Table 3. AK equipped with 12-carbon chain or less did not show toxicity up to tested concentrations while the AK with a carbon chain of fourteen or longer have shown significant toxicity at 100 μg/mL. These results suggest that it may be necessary to make compromises with less than optimal antifungal activities and cytotoxicities for selecting efficacious AK analogs (such as in the case of K20), for clinical and agricultural applications.

Treatments for fungal diseases can be challenging due to the emergence of drug-resistant fungi and the unclear distinction of fungal infections from bacterial infections. The latter can be difficult for physicians due to similar symptoms from bacteria and fungi. Nevertheless, in order to offer prompt therapeutic options, it is pivotal to identify the nature of the microbes that cause the infection since antifungal agents are, in general, inactive against bacteria and vice versa. Current practices for detection of fungi, such as culture method and PCR may take days to yield the result [10]. Amphiphilic kanamycin has been shown to exert a fast membrane permeabilization (within a few minutes) and endocytosis selective toward fungi over bacteria [11]. Therefore, we decided to synthesize the fluorescent AK, **7** as described in Scheme 1 for the potential application of offering prompt differentiation of fungi from bacteria. Two strains of fungi, *C. albicans* 64124 (azole-resistant) and *C. albicans* MYA2876 (azole susceptible), and two strains of bacteria, *E. coli* (G-) and *S. aureus* (G+) were selected for the imaging study. Fungi were treated with compound **7** at 32 μg/mL, which is about ¼ of the MIC. Bacteria were also treated with



Scheme 1. Synthesis of amphiphilic kanamycin.

Table 1
Antifungal activity of AK.[a].

Compound	A[b]	B[b]	C[b]	D[b]	E[b]	F[b]	G[b]	H[b]	I[b]	J[b]
3	>256	>256	>256	>256	>256	>256	>256	>256	128	128
4a	>256	>256	>256	>256	>256	>256	128	128	128	64
4b	256	>256	>256	>256	>256	>256	16	16	16	32
4c	128	>256	256	256	256	>256	8	8	8	32
4d	16	64	32	16	32	32	2	2	2	2
4e	4	8	8	4	4	4	2	2	2	2
4f	4	8	4	4	4	4	2	2	2	2
5a	>256	>256	>256	>256	>256	>256	128	64	32	256
5b	128	256	>256	256	>256	>256	128	64	32	128
5c	64	64	128	64	128	128	8	8	4	32
5d	16	16	32	16	32	32	2	2	2	4
5e	4	4	8	8	16	4	2	2	2	2
5f	4	4	4	4	4	4	2	2	2	2
7	128	256	ND[c]	ND	ND	ND	64	64	32	32
K20	16	16	16	32	16	16	4	4	4	8
Kanamycin A	≥256	≥256	≥256	ND	ND	ND	ND	≥256	≥256	≥256

[a] Unit: μg/mL.
[b] A: *C. albicans* 64124, B: *C. albicans* MYA2876, C: *C. albicans* B-311, D: *C. rugosa* 95–967, E: *C. parapsolis* Cas08-0490 (azole resistant), F: *C. tropicalis* 95-41, G: *C. neoformans* H99, H: *C. neoformans* VR-54, I: *R. pilimanae* (ATCC 26423), J: *F. graminearium* B4-5A.
[c] ND: Not determined.

compound **7** at 32 μg/mL, which is the MIC for *E. coli* and ½ of the MIC for *S. aureus*. The images were taken after 5 and 60 min of incubation with compound **7** (Fig. 3).

From the images, it is clear that compound **7** can quickly stain the fungal yeast Candida strains even with 5 min of incubation and at less than MIC concentrations. In contrast, no fluorescent staining can be observed for both bacteria (*E. coli* and *S. aureus*) after 5 min. Only after incubating for 60 min, did *E. coli* and *S. aureus* begin to

Table 2
MICs of AK against bacterial strains.[a].

Compound	A[b]	B[b]	C[b]	D[b]	Compound	A[b]	B[b]	C[b]	D[b]
3	16	32	>256	>256	**5a**	128	256	>256	>256
4a	256	64	>256	>256	**5b**	128	128	256	256
4b	256	128	>256	128	**5c**	64	64	64	32
4c	64	128	256	64	**5d**	16	32	32	16
4d	16	64	64	32	**5e**	16	16	16	16
4e	32	16	16	16	**5f**	32	16	32	16
4f	32	16	32	32	**7**	32	64	>256	>256
Kanamycin A	4	4	>256	>256	Vancomycin	ND[c]	ND	4	2

[a] Unit: μg/mL.
[b] A: *E. coli* (ATCC 25922), B: *S. aureus* (ATCC 25923), C: *S. aureus* (ATCC 33591) MRSA, D: S. *aureus* (ATCC 43300) MRSA.
[c] ND: Not determined.

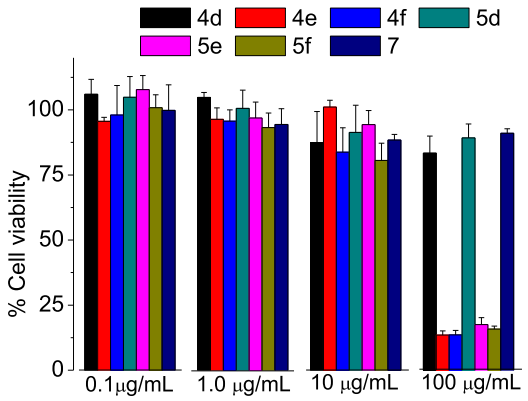


Fig. 2. Cytotoxicity of AK against HeLa cells.

Table 3
IC_{50} value of the AK against HeLa cells.[a].

Compound	IC_{50} value
4d	>100
4e	60.79 ± 3.13
4f	41.56 ± 10.23
5d	>100
5e	52.88 ± 5.78
5f	37.73 ± 7.33
7	>100

[a] Unit = μg/mL.

show slight fluorescent staining. Since the concentration of **7** employed was not fungicidal, but closer to bactericidal, the relatively strong fluorescence emitted from fungal cells when treated with compound **7** indicates that fluorescent AK analogs like compound **7** can be employed for differential diagnostic detection of fungi vs. bacteria at single-cell imaging resolution.

4. Conclusion

We have developed a practical and scalable route for the synthesis of 6″-modified AK that is suitable for the introduction of various connecting functionalities between the kanamycin core and hydrophobic moieties. Two libraries of AK bearing amine and thiol connecting functionalities were synthesized. Similar SARs show that tetradecyl (C14) and hexadecyl (C16) are the optimal chain length to exert broad-spectrum antifungal activity and modest antibacterial activity. Little or no difference in bioactivity displayed by the two libraries containing amine or thiol connecting functionalities suggests a minor role of these functionalities. However, the cytotoxicity evaluation revealed that as chain length increases (≥C14), cytotoxicity also increases. Combining these results, we conclude that AK equipped with longer chain length exert superior bioactivity against fungi and bacteria, but are associated with increased cytotoxicity, making these AKs behave more like non-selective antiseptic agents. In contrast, **K20** developed previously in our group represents one of the few examples of AK that best compromises between antifungal/antibacterial activity and toxicity to mammalian cells; it is selectively active toward fungi but inactive against bacteria or mammalian cells. The synthesis of fluorescent compound **7** and its fungal selective properties may pave the way for the development of diagnostic tools that can promptly differentiate fungal infection from diseases of bacterial origin.

5. Experimental section

Materials and Methods. Chemicals purchased from the commercial source were used without further purification unless otherwise mentioned. Pyridine was dried by keeping over calcium hydride for 72 h and recycled for further use. DMF was dried by treating with a molecular sieve for 72 h before using for the reaction. ^{1}H NMR, ^{1}H−^{1}H COSY, and ^{13}C NMR spectra of the compounds were obtained using a Brucker AvanceIII HD Ascend-500 at 500 MHz and 125 MHz respectively. Multiplicity of the peaks is reported as s = singlet, d = doublet, t = triplet, m = multiplet, b = broad. Coupling constants are presented in the hertz (Hz). Emission and excitation spectra of **7** were recorded in SHIMADZu RF-5301PC Spectrofluorophotometer. Emission wavelength was fixed at 520 nm while scanning excitation wavelength and the compounds were excited at 480 nm for emission spectra of **7**.

General Procedure for the Synthesis of compound 4a - 4f. To a solution of **2** (0.104 g, 1 equiv, 0.1 mmol) dissolved in anhydrous dioxane, 3 equiv of alkyl amine was added and the reaction mixture was refluxed for 24 h. Solvent was removed under reduced pressure and the solid residue was washed with 3 × 10 mL of water. During the washing, 10 mL of water was added to the solid, stirred for 15 min, and then filtered. This process was repeated two more times before moving to the next step. After washing, the reaction mixture was loaded onto a silica gel column and eluted with solvents ranging from 100% DCM to 10% MeOH in DCM. The eluted fractions were analyzed by TLC (5% methanol in DCM), and those that contained desired products were combined and concentrated to provide a yellowish white precipitate. The collected precipitate was re-dissolved in anhydrous DCM (10 mL) and trifluoroacetic acid (TFA) (1 mL) was added. After being stirred for 6 h, solvents were removed, and the residual reaction mixture was loaded onto a column packed with Amberlite CG50 ion exchange resin, and eluted with a gradient of solvent (100% water to 20% NH_4OH in water). Pure compound obtained after CG50 purification was further subjected to the ion exchange using anion exchange resin IR410 (Cl^-

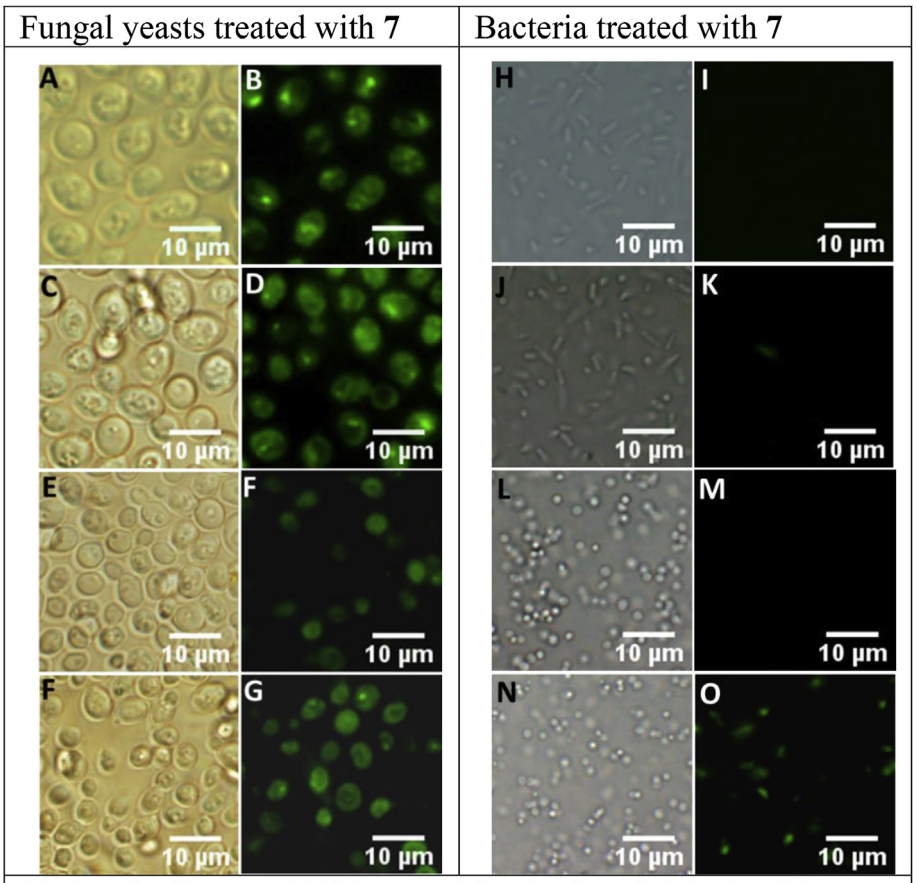


Fig. 3. Images of fungi and bacteria incubated with **7**.

form) to yield the desired amphiphilic kanamycin as a chloride salt.

General Procedure for the Synthesis of 5a - 5f. To a solution of **2** (0.104 gm, 1 equiv, 0.1 mmol) dissolved in dimethylformade (DMF), 5 equiv of alkylthiol and 5 equiv of cesium carbonate was added and the reaction mixture was stirred for 48 h at room temperature. Solvent was removed, and the precipitate was washed with water 4 × 20 mL of water using a Buchner funnel. During the washing, 20 mL of water was added to the precipitate, stirred for 15 min, and then filtered. This process was repeated three more times. The precipitate was further washed with 3 × 10 mL of hexane. The residual solid was dissolved in 10 mL of anhydrous DCM and 1 mL of TFA was added, and stirred at room temperature for 6 h. Solvents were removed, and the residual reaction mixture was loaded onto a column packed with Amberlite CG50 ion exchange resin, and eluted with a gradient of solvent (100% water to 20% NH_4OH in water). Pure compound obtained after CG50 purification was further subjected to the ion exchange using anion exchange resin IR410 (Cl^- form) to yield the desired amphiphilic kanamycin as a chloride salt.

6″-*O*-Toluenesulfonyl-1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl) kanamycin A (2). A solution of 1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl)kanamycin A (86.9 gm, 1 equiv, 100 mmol) was dissolved in 1200 mL of anhydrous pyridine by magnetic stirring at room temperature. The reaction flask was then transferred to the ice bath and stirred for 15 min. A solution of toluenesulfonyl chloride (TsCl) (47.67 gm, 2.5 equiv, 250 mmol) dissolved in 300 mL of anhydrous pyridine was slowly added to the stirring solution of 1,3,6′,3″-tetra-*N*-(*tert*-butoxycarbonyl)kanamycin A at 0 °C over the period of an hour. After completion of the addition of TsCl, the reaction mixture was stirred for additional 30 min. The ice bath was removed and the reaction mixture was stirred for 3 days allowing the temperature to rise slowly to room temperature. The reaction was quenched by addition of methanol (6 mL, 1.5 equiv, 0.15 mol). After being stirred for 30 min, the solvents were removed using a rotary evaporator under reduced pressure while keeping the temperature of the water bath below 30 °C. The viscous residue obtained after the removal of the solvent solidified into powder and was washed with 5 × 500mL water using a Buchner funnel. The resulting pale yellowish powder was loaded in to the silica gel column and purified by eluting with a gradient of solvent (100% DCM to 5% MeOH in DCM). The compound **2** was obtained as a white solid (56.4 g, 6.4 mmol, 54%). ^1H NMR (500 MHz, CD_3OD)

δ 7.79 (d, $J = 8.0$ Hz, 2H), 7.45 (d, $J = 8.0$ Hz, 2H), 5.08 (b, 1H), 5.00 (b, 1H), 4.1–4.4 (m, 3H), 3.3–3.8 (m, 12H), 3.03 (t, $J = 9.5$ Hz, 1H), 2.48 (s, 3H), 2.0–2.1 (m, 1H), 1.4–1.5 (m, 37H); ^{13}C NMR (125 MHz, CD_3OD) δ 157.97, 157.82, 156.54, 156.28, 132.99, 129.63 (2C), 127.72 (2C), 101.41, 98.38, 84.34, 79.91, 79.22, 79.01 (2C), 78.78, 75.33, 73.10, 72.52, 70.95, 70.45, 70.33, 70.21, 69.05, 67.71, 55.93, 50.60, 49.37, 40.49, 34.63, 27.53 (3C), 27.49 (3C), 27.45 (6C), 20.31. ESI/APCI calcd for $C_{45}H_{74}N_4NaO_{21}S^+$ $[MNa]^+$: 1061.4464; measured m/e: 1061.4474.

6″-*O*-Toluenesulfonylkanamycin A (3). To a solution of **2** (0.104 gm, 0.1 mmol) dissolved in anhydrous DCM (5 mL), trifluoroacetic acid was added (0.5 mL). After being stirred at room temperature for 6 h, the solvent was removed. The crude product was subjected to ion exchange using anion exchange resin IR410 (Cl^- form) to yield the desired amphiphilic kanamycin as a chloride salt. The compound was synthesized as white solid (74.5 mg, 0.095 mmol, 95%). ^{1}H NMR (500 MHz, D_2O) δ 7.72 (d, $J = 8.5$ Hz, 2H), 7.37 (d, $J = 8.5$ Hz, 2H), 5.49 (d, $J = 4.0$ Hz, 1H), 4.94 (d, $J = 3.5$ Hz, 1H), 4.2–4.3 (m, 2H), 4.0–4.1 (m, 1H), 3.9–4.0 (m, 1H), 3.2–3.9 (m, 12H), 3.1–3.2 (m, 1H), 2.4–2.5 (m, 1H), 2.34 (s, 3H), 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 146.75, 130.37, 130.24 (2C), 127.93 (2C), 100.55, 96.25, 83.82, 78.20, 72.53, 72.08, 70.82, 70.81, 70.33, 69.06, 68.69, 67.90, 65.19, 54.72, 49.93, 47.89, 40.43, 27.58, 20.97. ESI/APCI calcd for $C_{25}H_{43}N_4O_{13}S^+$ $[MH]^+$: 639.2547; measured m/e: 639.2543.

6″-(1-Hexylamino)-6″-deoxykanamycin A (4a). The compound was synthesized according to the general procedure and obtained as a white solid (41.9 mg, 0.056 mmol, 56%). ^{1}H NMR (500 MHz, D_2O) δ 5.75 (d, $J = 4.0$ Hz, 1H), 5.08 (d, $J = 3.5$ Hz, 1H), 4.1–4.2 (m, 1H), 3.3–4.0 (m, 15H), 3.1–3.2 (m, 1H), 3.03 (t, $J = 9.0$ Hz, 2H), 2.4–2.5 (m, 1H), 1.8–1.9 (m, 1H), 1.6–1.7 (m, 2H), 1.2–1.4 (m, 6H), 0.79 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.4, 98.5, 83.7, 79.7, 74.1, 71.8, 70.9, 70.6, 68.7, 68.2, 68.0, 67.2, 54.6, 50.1, 48.4, 48.3, 47.7, 40.2, 30.4, 28.3, 25.3 (2C), 21.7, 13.2. ESI/APCI calcd for $C_{24}H_{50}N_5O_{10}^+$ $[MH]^+$: 568.3558; Measured m/e: 568.3576.

6”-(1-Octylamino)-6″-deoxykanamycin A (4b). The compound was synthesized according to the general procedure and obtained as a white solid (35.0 mg, 0.045 mmol, 45%). ^{1}H NMR (500 MHz, D_2O) δ 5.44 (d, $J = 4.0$ Hz, 1H), 5.03 (d, $J = 3.5$ Hz, 1H), 4.1–4.2 (m, 1H), 3.1–4.0 (m, 16H), 3.02 (t, $J = 8.0$ Hz, 2H), 2.2–2.3 (m, 1H), 1.5–1.7 (m, 3H), 1.1–1.3 (m, 10H), 0.78 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.37, 98.44, 83.72, 79.69, 74.18, 71.84, 70.00, 70.75, 68.71, 68.25, 68.04, 67.21, 54.64, 50.13, 48.42, 48.36, 47.71, 40.33, 31.01, 28.35, 28.20, 28.18, 25.66, 25.37, 21.79, 13.40. ESI/APCI calcd for $C_{26}H_{54}N_5O_{10}$: 596.3871; measured m/e: 596.3864.

6″-(1-Decylamino)-6″-deoxykanamycin A (4c). The compound was synthesized according to the general procedure and obtained as a white solid (47.6 mg, 0.059 mmol, 59%). ^{1}H NMR (500 MHz, D_2O) δ 5.57 (d, $J = 4.0$ Hz, 1H), 5.07 (d, $J = 4.0$ Hz, 1H), 4.1–4.2 (m, 1H), 3.9–4.0 (m, 2H), 3.7–3.9 (m, 4H), 3.3–3.6 (m, 7H) 3.1–3.2 (2H), 3.0–3.1 (t, $J = 8.0$ Hz 2H), 2.4–2.5 (m, 1H), 1.7–1.8 (m, 1H), 1.6–1.7 (m, 2H), 1.2–1.3 (m, 14H), 0.78 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.39, 98.52, 83.73, 79.78, 74.19, 71.86, 71.01, 70.75, 68.73, 68.26, 68.05, 67.21, 54.65, 50.14, 48.42, 48.36, 47.72, 40.34, 31.16, 28.65, 28.51, 28.45, 28.35, 28.24, 25.67, 25.38, 22.03, 13.42. ESI/APCI calcd for $C_{28}H_{58}N_5O_{10}^+$ $[MH]^+$: 624.4184; measured m/e: 624.4181.

6”-(1-Dodecylamino)-6″-deoxykanamycin A (4d). The compound was synthesized according to the general procedure and obtained as a white solid (54.2 mg, 0.065 mmol, 65%). ^{1}H NMR (500 MHz, D_2O) δ 5.52 (d, $J = 3.5$ Hz, 1H), 5.07 (d, $J = 3.5$ Hz, 1H), 4.1–4.2 (m, 1H), 3.8–4.0 (m, 2H), 3.6–3.8 (m, 4H), 3.2–3.6 (m, 8H), 3.1–3.2 (m, 2H), 3.0–3.1 (m, 2H), 2.2–2.3 (m, 1H), 1.5–1.7 (m, 3H), 1.2–1.3 (m, 18H), 0.77 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.25, 98.78, 84.23, 81.56, 74.38, 72.00, 71.13, 70.84, 68.64, 68.23, 68.14, 67.44, 54.68, 50.40, 48.58, 48.33, 47.75, 40.38, 31.18, 28.77 (3C), 28.69, 28.52, 28.50, 28.42, 25.68, 25.38, 22.03, 13.42. ESI/APCI calcd for $C_{30}H_{62}N_5O_{10}^+$ $[MH]^+$: 652.4497; measured m/e: 652.4487.

6”-(1-Tetradecylamino)-6″-deoxykanamycin A (4e). The compound was synthesized according to the general procedure and obtained as a white solid (40.5 mg, 0.047 mmol, 47%). ^{1}H NMR (500 MHz, D_2O) δ 5.56 (d, $J = 3.5$ Hz, 1H), 5.06 (d, $J = 3.5$ Hz, 1H), 4.0–4.2 (m, 1H), 3.9–4.0 (m, 2H), 3.6–3.8 (m, 4H), 3.2–3.6 (m, 8H), 3.1–3.2 (m, 2H), 3.0–3.1 (m, 2H), 2.2–2.3 (m, 1H), 1.7–1.8 (m, 1H), 1.6–1.7 (m, 2H), 1.1–1.3 (m, 22H), 0.78 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.43, 98.70, 83.74, 79.93, 74.20, 71.87, 70.99, 70.70, 68.75, 68.29, 68.04, 67.19, 54.64, 50.12, 48.39, 48.34, 47.69, 40.30, 31.19, 28.78 (5C), 28.69, 28.50 (2C), 28.25, 25.68, 25.37, 22.03, 13.41. ESI/APCI calcd for $C_{32}H_{66}N_5O_{10}^+$ $[MH]^+$: 680.4810; measured m/e: 680.4810.

6”-(1-Hexadecylamino)-6″-deoxykanamycin A (4f). The compound was synthesized according to the general procedure and obtained as a white solid (17.8 mg, 0.020 mmol, 20%). ^{1}H NMR (500 MHz, CD_3OD) δ 5.58 (d, $J = 3.5$ Hz, 1H), 5.22 (d, $J = 3.5$ Hz, 1H), 4.2–4.3 (m, 1H), 4.0–4.1 (m, 1H), 3.7–3.9 (m, 4H), 3.4–3.7 (m, 7H), 3.0–3.3 (m, 6H), 2.4–2.5 (m, 1H), 1.7–1.9 (m, 3H), 1.3–1.4 (m, 26H), 0.92 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 100.29, 97.75, 84.59, 81.29, 73.42, 72.65, 71.87, 71.66, 69.00, 68.76, 68.56, 67.80, 55.12, 50.67, 48.65, 48.39, one carbon peak underneath the solvent peak, 40.72, 31.66, 29.40 (6C), 29.36, 29.32, 29.21, 29.07, 28.89, 26.28, 25.64, 22.32, 13.03. ESI/APCI calcd for $C_{34}H_{70}N_5O_{10}^+$ $[MH]^+$: 708.5123, measured m/e: 708.5121.

6″-(1-Hexylmercapto)-6″-deoxykanamycin A (5a). The compound was synthesized according to the general procedure and obtained as a white solid (51.8 mg, 0.071 mmol, 71%). ^{1}H NMR (500 MHz, D_2O) δ 5.52 (d, $J = 4.0$ Hz, 1H), 5.01 (d, $J = 4.0$ Hz, 1H), 3.8–4.0 (m, 5H), 3.6–3.8 (m, 2H), 3.2–3.6 (m, 7H), 3.1–3.2 (m, 1H), 2.9–3.0 (m, 1H), 2.6–2.7 (m, 1H), 2.53 (t, $J = 7.5$ Hz, 2H) 2.3–2.4 (m, 1H), 1.7–1.8 (m, 1H), 1.4–1.5 (m, 2H), 1.1–1.3 (m, 6H), 0.76 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.33, 96.70, 84.34, 78.68, 73.19, 72.19 (2C), 70.88, 70.78, 68.59, 68.44, 68.21, 54.82, 49.78, 47.98, 40.38, 32.41, 32.39, 30.63, 28.79, 28.31, 27.64, 21.89, 13.38. ESI/APCI calcd for $C_{24}H_{49}N_4O_{10}S^+$ $[MH]^+$: 585.3164; measured m/e: 585.3171.

6”-(1-Octylmercapto)-6″-deoxykanamycin A (5b). The compound was synthesized according to the general procedure and obtained as a white solid (40.9 mg, 0.054 mmol, 54%). ^{1}H NMR (500 MHz, D_2O) δ 5.54 (d, $J = 4.0$ Hz, 1H), 5.03 (d, $J = 3.5$ Hz, 1H), 3.8–4.0 (m, 5H), 3.7–3.8 (m, 2H), 3.5–3.6 (m, 4H), 3.2–3.5 (m, 3H), 3.1–3.2 (m, 1H), 2.9–3.0 (m, 1H), 2.6–2.7 (m, 1H), 2.56 (t, $J = 7.5$ Hz, 2H), 2.4–2.5 (m, 1H), 1.8–1.9 (m, 1H), 1.5–1.6 (m, 2H), 1.1–1.4 (m, 10H), 0.78 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.46, 96.48, 84.11, 78.03, 72.95, 72.20, 72.11, 70.80, 70.72, 68.65, 68.38, 68.17, 54.80, 49.69, 47.83, 40.35, 32.41, 32.37, 31.10, 28.81, 28.34, 28.28, 27.95, 27.59, 22.01, 13.42. ESI/APCI Calcd for $C_{26}H_{53}N_4O_{10}S^+$ $[MH]^+$: 613.3482; measured m/e: 613.3486.

6”-(1-Decylmercapto)-6″-deoxykanamycin A (5c). The compound was synthesized according to the general procedure and obtained as a white solid (35.4 mg, 0.045 mmol, 45%). ^{1}H NMR (500 MHz, D_2O) δ 5.52 (d, $J = 4.0$ Hz, 1H), 5.02 (d, $J = 3.5$ Hz, 1H), 3.8–4.0 (m, 5H), 3.6–3.8 (m, 3H), 3.2–3.6 (m, 7H), 3.1–3.2 (m, 1H), 2.9–3.0 (m, 1H), 2.6–2.7 (m, 1H), 2.55 (t, $J = 7.5$ Hz, 2H) 2.3–2.4 (m, 1H), 1.7–1.8 (m, 1H), 1.5–1.6 (m, 2H), 1.1–1.3 (m, 14H), 0.78 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.33, 96.88, 84.49, 79.10, 73.30, 72.16, 72.13, 70.91, 70.78, 68.59, 68.46, 68.23, 54.82, 49.83, 48.02, 40.37, 32.42, 32.39, 31.18, 28.80, 28.70, 28.65 (2C), 28.48, 28.30, 27.93, 22.04, 13.43. ESI/APCI calcd for $C_{28}H_{57}N_4O_{10}S^+$ $[MH]^+$: 641.3795; measured m/e: 641.3789.

6”-(1-Dodecylmercapto)-6″-deoxykanamycin A (5d). The

compound was synthesized according to the general procedure and obtained as a white solid (49.7 mg, 0.061 mmol, 61%). ^{1}H NMR (500 MHz, D_2O) δ 5.49 (d, J = 3.5 Hz, 1H), 4.99 (d, J = 3.5 Hz, 1H), 3.8–4.0 (m, 4H), 3.2–3.7 (m, 10H), 3.0–3.1 (m, 1H), 2.9–3.0 (m, 1H), 2.6–2.7 (m, 1H), 2.52 (t, J = 7.5 Hz, 2H), 2.3–2.4 (m, 1H), 1.6–1.7 (m, 1H), 1.4–1.6 (m, 2H), 1.1–1.3 (m, 18H), 0.75 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 100.27, 97.08, 84.65, 79.61, 73.45, 72.17, 72.09, 70.96, 70.80, 68.56, 68.49, 68.27, 54.82, 49.87, 48.10, 40.36, 32.44 (2C), 31.22, 28.82 (4C), 28.76, 28.68, 28.54, 28.34, 27.97, 22.06, 13.44. ESI/APCI calcd for $C_{30}H_{61}N_4O_{10}S^+$ $[MH]^+$: 669.4108; measured m/e: 669.4107.

6"-(1-Tetradecylmercapto)-6″-deoxykanamycin A (5e). The compound was synthesized according to the general procedure and obtained as a white solid (36.2 mg, 0.043 mmol, 43%). ^{1}H NMR (500 MHz, CD_3OD) δ 5.61 (d, J = 4.0 Hz, 1H), 5.13 (d, J = 3.5 Hz, 1H), 4.0–4.1 (m, 2H), 3.2–4.0 (m, 12H), 3.0–3.1 (m, 2H), 2.6–2.8 (m, 3H), 2.4–2.5 (m, 1H), 1.8–2.0 (m, 1H), 1.5–1.7 (m, 2H), 1.2–1.5 (m, 22H), 0.92 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 100.41, 95.59, 85.34, 79.29, 73.07 (2C), 72.57, 71.68, 71.64, 68.90, 68.85, 68.63, 55.27, 50.14, one peak underneath the solvent peak, 40.81, 32.77, 32.67, 31.67, 29.49, 29.40 (3C), 29.37 (3C), 29.23, 29.07 (2C), 28.59, 22.33, 13.03. ESI/APCI Calcd for $C_{32}H_{65}N_4O_{10}S^+[MH]^+$: 697.4421; measured m/e: 697.4425..

6"-(1-Hexadecylmercapto)-6″-deoxykanamycin A (5f). The compound was synthesized according to the general procedure and obtained as a white solid (27.0 mg, 0.031 mmol, 31%). ^{1}H NMR (500 MHz, CD_3OD) δ 5.50 (d, J = 3.5 Hz, 1H), 5.02 (d, J = 3.5 Hz, 1H), 3.9–4.0 (m, 2H), 3.1–4.0 (m, 12H), 2.9–3.0 (m, 2H), 2.5–2.7 (m, 3H), 2.3–2.4 (m, 1H), 1.7–1.8 (m, 1H), 1.4–1.6 (m, 2H), 1.1–1.4 (m, 26H), 0.80 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 100.42, 95.56, 85.26, 79.17, 73.06 (2C), 72.57, 71.69, 71.65, 68.88, 68.85, 68.63, 55.28, 50.13, one peak underneath the solvent peak, 40.82, 32.77, 32.67, 31.67, 29.49, 29.39 (6C), 29.35 (2C), 29.07 (3C), 28.59, 22.33, 13.04. ESI/APCI calcd for $C_{34}H_{69}N_4O_{10}S^+[MH]^+$: 725.4734; measured m/e: 725.4730.

6″-(2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl))benzamido)-6″-deoxykanamycin A (7). To a solution of **2** (1.04 gm, 1 equiv, 1.00 mmol) dissolved in 20 mL of anhydrous DMF, sodium azide (0.214, 3.3 equiv, 3.3 mmol) was added, and the reaction was stirred at 80 °C under nitrogen atmosphere. After being stirred for 48 h, solvent was removed under reduced pressure and the solid residue was washed with water 3 × 10 mL. The washed precipitate was dried under vacuum for 24 h. The dried crude compound was then dissolved in 10 mL of 1:1 solution of degassed MeOH and AcOH. Catalytic amount of 10% Pd/C was added and the reaction mixture was stirred at room temperature for 12 h under hydrogen gas environment. The reaction mixture was filtered through Celite and the solvents were removed. The solid residue was washed with 2 × 5 mL of 10% sodium carbonate aqueous solution. The residue was further washed with 2 × 5 mL of DCM to obtain compound **6** [12]. For a solution of compound **6** (0.100 gm, 1 equiv, 0.11 mmol) dissolved in 10 mL of DMF, NHS activated fluorescein (0.095 gm, 2 equiv, 0.22 mmol) [13], was added. The reaction mixture was stirred for 24 h at room temperature. Solvent was removed, and the viscous residue was washed with 2 × 5 mL water and dried under vacuum. The dried precipitate was treated with a solution of 5 mL of anhydrous DCM and 1 mL of trifluoro acetic acid, and the reaction mixture was stirred for 6 h at room temperature. After removal of solvents, the viscous residue was loaded to a column packed with by Amberlite CG50 and eluted with a gradient of solvent (100% water to 20% NH_4OH in water). Pure compound obtained after CG50 purification was further subjected to the ion exchange using anion exchange resin IR410 (Cl^- form) to yield the desired amphiphilic kanamycin as a chloride salt (24.0 mg, 0.025 mmol, 23%). ^{1}H NMR (500 MHz, D_2O) δ 7.8–7.9 (m, 1H), 7.5–7.6 (m, 2H), 6.9–7.0 (m, 1H), 6.6–6.7 (m, 2H), 6.55 (d, J = 9.0 Hz, 1H), 6.4–6.5 (m, 2H), 6.40 (d, J = 8.5 Hz, 1H), 5.45 (d, J = 3.5 Hz, 1H), 4.60 (d, J = 3.5 Hz, 1H), 3.7–3.9 (m, 3H), 3.6–3.7 (m, 2H), 3.56 (t, J = 10.0 Hz, 1H), 3.3–3.5 (m, 7H), 3.1–3.3 (m, 3H), 2.8–2.9 (m, 1H), 2.4–2.5 (m, 1H), 1.8–1.9 (m, 1H); ^{13}C NMR (125 MHz, D_2O) δ 171.74, 157.40, 156.99, 153.04, 152.92, 152.77, 134.31, 130.09, 129.61, 129.35, 129.32, 124.24, 122.83, 112.56, 112.08, 111.36, 109.21, 102.65, 102.53, 100.24, 96.26, 84.29, 76.28, 73.96, 72.15, 71.97, 70.67, 70.63, 68.62, 67.30, 66.37, 66.27, 53.68, 49.12, 48.26, 41.05, 40.05, 27.47. ESI/APCI calcd for $C_{38}H_{48}N_5O_{14}^+$ $[MH]^+$: 798.3198; measured m/e: 798.3165.

Procedure for the antifungal activity. Minimum inhibitory concentrations of the **c**ompounds were evaluated by two-fold dilution method following standard protocol [14,15]. All the fungal strains were grown in RPMI medium supplement with 0.165 M MOPS buffer (pH = 7) at 28 °C with gentle shaking for 48 h. Compounds were dissolved in water to make stock solution of 10 mg/mL. The compounds from stock were diluted in 96 well plate using growth medium to the final concentration ranging from 512 to 0.25 μg/mL 50 μL of fungi with recommended confluence from the standard protocol was added to each well containing 50 μL of diluted compounds and incubated for 24–36 h. The minimum concentration of compound that inhibits fungal growth to visible clearance is reported as the MIC value.

Procedure for the antibacterial activity. Bacterial strains were grown in Lysogeny broth (LB) at 35 °C. MIC of compounds against was also determined by two-fold dilution method. The compounds were diluted in a 96-well plate using water to the final concentration from 512 to 0.25 μg/mL 50 μL of bacteria growth with adjusted confluence (OD_{600} = 0.08–0.1) was added to each well of the 96-well plate with 50 μL of diluted compounds. The plate was incubated for 18 h at 35 °C and read visually. The minimum concentration of compound that inhibited the bacterial growth to visible clearance is reported as the MIC value.

Procedure for the evaluation of cytotoxicity towards human cell. Cytotoxicity of the compounds was evaluated by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. The compounds' cytotoxicity towards human cells was evaluated against the HeLa cell, a cervical human cell. Cells were grown in DMEM medium (Corning) supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin under 5% CO_2 in humid environment at 37 °C. 200 μL of cell suspension was added to each well of a 96-well plate from 25,000 cells/mL stock and incubated for 24 h. The medium was removed, and the compounds were? diluted in growth medium from 100 μg/mL - 0.1 μg/mL with ten-fold dilution along with just medium was added to the cells. After 24 h of incubation, 20 μL of MTT dye (5 mg/mL in PBS) was added and incubated for 5 more hrs. The medium was removed, and the reduced formazan dye was dissolved in 200 μL of DMSO. Absorption of formazan solution was measured at 570 nm with 650 nm as the background absorption. The experiment was done in triplicate of triplicate. During the analysis, absorption from the control was converted to 100% cell viability and the cell viability at different concentration of the compounds were calculated relative to the control.

Procedure for the imaging of fungi incubated with compound 7. Fungi were grown in the PDB medium with gentle shaking at 28 °C for 48 h. 1 mL of fungal growth was transferred to the centrifuge tube and spun down at 10,000 rpm for 2 min in a Fisher Scientific accuSpin MicroCentrifuge at room temperature. Cell pallets were washed one more time using the same volume of the water. The fungal cells were then incubated with 1 mL of 32 μg/mL of compound **7** for 5 or 60 min. The final confluence of fungal cells was maintained to 1 × 107 cells/mL during the incubation with compound **7**. After the incubation with compound **7**, fungal cells were washed with the same volume of water by centrifugation and

resuspended in 1 mL of water. Images of fungi were taken using an Olympus IX71 microscope using 100X oil immersion objective under phase contrast and green channel.

Procedure for the imaging of bacteria incubated with compound 7. Bacteria were grown in LB medium at 37 °C for 18 h 1 mL of bacteria in each centrifuge tube was spun down at 10,000 rpm for 2 min in a Fisher Scientific accuSpin Micro at room temperature. Bacterial cells were washed with water twice using same volume of water by centrifugation. Bacterial cells were then treated with 1 mL of 32 μg/mL of compound **7**. After 5 or 60 min of incubation, cells were washed with water twice. After washing, cells were resuspended in 100 μL of water. Images were taken in Olympus IX71 microscope using 100X oil immersion objective under phase contrast and green channel.

Author contributions

Alkyl AKs were synthesized and characterized by Y.P.S., U.P., H.M., P. R., J.W., G. N. and antifungal assays were performed by Y.P.S., M.N.A., M.G. Synthesis and imaging with the fluorescent analog, antibacterial activity, cytotoxicity were performed and analyzed by Y.P.S. The manuscript was written and edited by C.-W. T. C., Y.P.S, J.Y.T. and M.G.

Acknowledgements

We acknowledge support from the Department of Chemistry and Biochemistry, Utah State University and the Utah Science,-Technology and Research (USTAR) initiative, State of Utah. This work was supported in part by NSF Award CHE-1429195 for a 500 MHz Bruker NMR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2019.111639.

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