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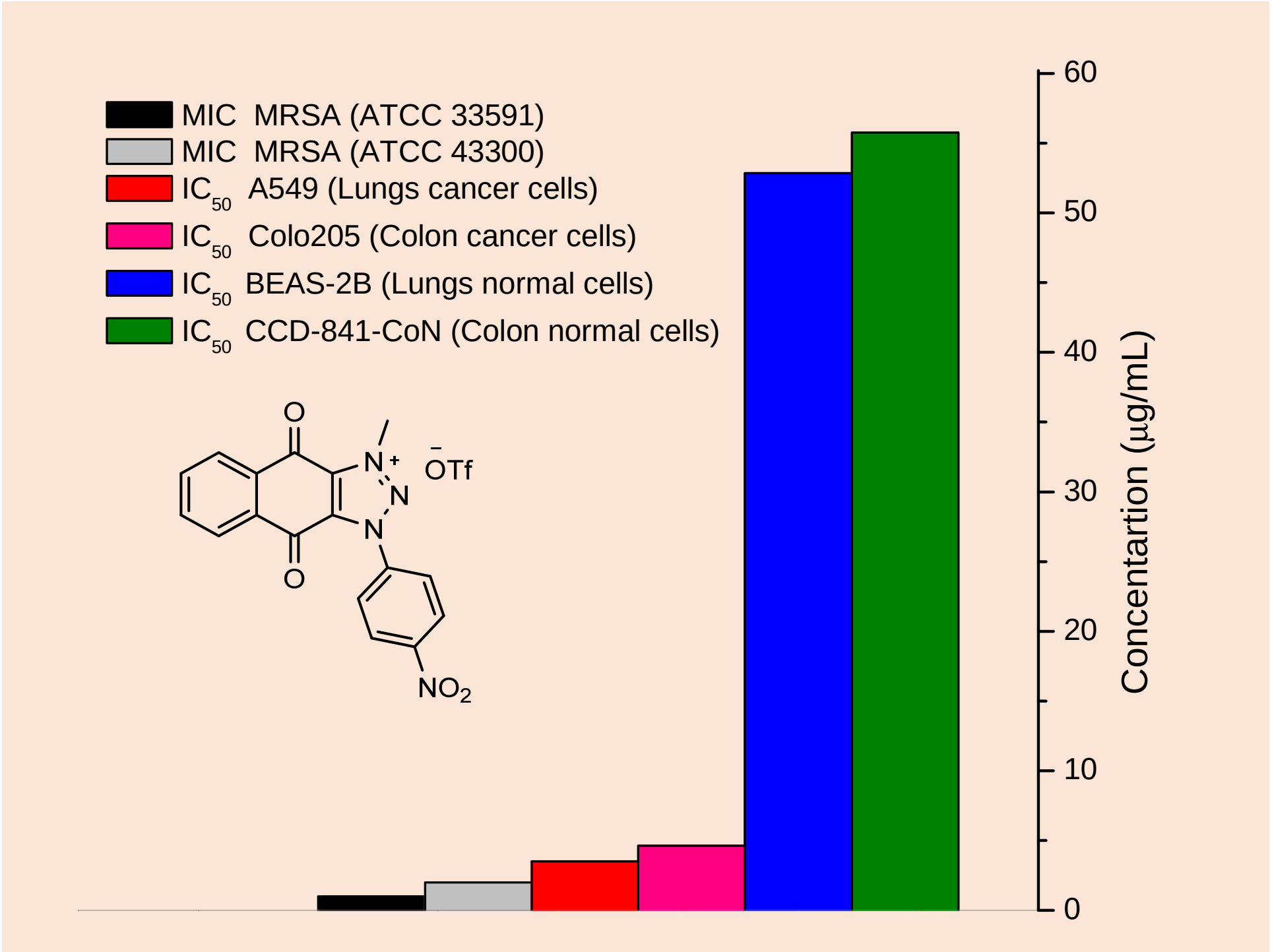

# Tuning the Biological Activity of Cationic Anthraquinone Analogues Specifically toward *Staphylococcus aureus*

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**Abstracts**

Development of new antibacterial agents against drug resistant bacteria is an imminent task, especially against methicillin-resistant *Staphylococcus aureus* (MRSA). While MRSA can still be treated with broad spectrum antibiotics, the use of which often leads to the disruption of normal microbial flora leading to *Clostridium difficile* infection (CDI). Herein, a new class of antibacterial agent, cationic anthraquinone analogues specifically against MRSA, has been developed. Through the variation and optimization of substituents, these agents are selective toward MRSA, and not Gram negative bacteria which may avoid the problem of CDI. In addition, newly discovered lead compounds also show significantly reduced cytotoxicity against normal mammalian cells than cancerous cells. This interesting finding can alleviate the toxicity and side effect problems often associate with the use of antibiotics.

**Key words**

cationic anthraquinone analogues, antibiotic, MRSA, vancomycin-resistant *Staphylococcus aureus* (VRSA), *Clostridium difficile* infection (CDI), cytotoxicity

## Introduction

Infectious diseases caused by pathogenic and opportunistic Gram positive (G+) bacteria remain a severe public health problem. Among these bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA)[1-3] and vancomycin-resistant *Staphylococcus aureus* (VRSA)[4] are of particular interest due to their prevalence in community, multi-drug resistance to many clinically used antibiotics, and the severity of illness caused by these bacteria. The burden of MRSA is significant. In 2009, there were an estimated 463,017 MRSA-related hospitalizations in the United States [5]. Although VRSA infection is still rare, a more serious concern is the transfer of

vancomycin resistance from common bacteria, such as *Enterococci*, to MRSA, leading to the increase in the VRSA infection [6, 7].

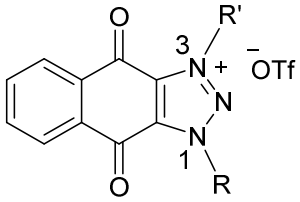
While MRSA can still be successfully treated with vancomycin, linezolid, daptomycin, teicoplanin, quinupristine-dalfopristine, and tigecycline, resistance against these antibacterial agents has been noted or reported. Approved by the FDA in 2003, daptomycin (Cubicin ®) is the latest novel antibiotic[8], however, drug-resistance has been observed in MRSA in 2005 [9], and reduced susceptibility has been reported in VRSA in 2006 [10]. Approved in 2001 by FDA, linezolid (Zyvox®) is one of the few clinically used antibiotics for treating enterococcus infection, however, linezolid drug-resistance by enterococci was noted in 2007 [11].

On the other hand, a common practice for treatment of bacterial infection is to apply broad-spectrum antibiotics, such as ciprofloxacin, imipenem and cefepime. Nevertheless, the use of the broad-spectrum antibiotics often eradicates beneficial bacteria, disrupts the normal microbial flora, and leads to the over-growth of *Clostridium difficile* and *C. difficile* infection (CDI) [12, 13]. CDI can range in severity from asymptomatic to severe and life threatening. In the United States alone, CDI increases the cost of healthcare by over 1 billion each year [14]. In short, these reports exemplify the need for the development of new antibiotics *selectively* against G+ bacterial pathogens, like MRSA. Finally, it is also important that the new antibiotics manifest low cytotoxicity toward normal mammalian cells to alleviate the problem of side effects.

**Rationale and Design**

In an effort to develop countermeasure against MRSA, our group has been studying the use of cationic anthraquinone analogues (CAAs) as antibacterial agents (Table 1) [15-17]. These antibacterial CAAs have two mode of actions (MOAs): (1) by disrupting the redox processes of bacteria or (2) by acting as membrane disrupting agents. The redox processes of bacteria can occur at the bacteria membrane, a site that can be accessible easier by CAAs. The latter MOA requires the attachment of relatively long linear alkyl chains (C8-C16) [18]. In general, these CAAs regardless of the type of structural modifications (*N*-1 and *N*-3 positions) all exert significant antibacterial activity against drug-susceptible *S. aureus* and MRSA with minimum inhibitory concentrations (MICs) less than 2 μg/mL. The activity against Gram negative (G-) bacteria, such as *Escherichia coli*, varies drastically upon structural modifications of CAAs. For example, most of the CAAs bearing alkyl groups at *N*-1 and *N*-3 positions (**1a**, **1b** and **1c**) are more active than those with aryl groups at *N*-1 positions (**2b**, **2c** and **2d**). This finding prompts our interest in the development of selective antibiotic against MRSA.

Table 1. Antibacterial activity (MIC) of CAAs[a]

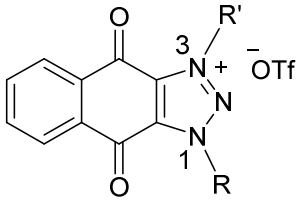



| Entry | Compound | R/R′ | *E. coli* (ATCC25922) | *S. aureus* (ATCC25923) | *S. aureus* MRSA (ATCC 33591) |
|---|---|---|---|---|---|
| 1 | **1a**[b] | Me/Me | 32-64 | 4-8 | 4-8 |
| 2 | **1b**[b] | $C_8H_{17}$/Me | 8 | 0.032-0.064 | 1 |
| 3 | **1c**[b] | $C_{16}H_{33}$/Me | 0.5-1 | 0.125-0.25 | 0.25 – 0.5 |
| 4 | **1d**[c] | C8H17/Et | >128 | 0.25 – 0.5 | - |
| 5 | **2a**[b] | Bn/Me | 8-16 | 2 | 0.5 - 1 |
| 6 | **2b**[d] | 4-MeOPh/Me | >128 | 1-2 | 0.5 |
| 7 | **2c**[d] | Ph/Me | 128 | 1 | 0.5 |
| 8 | **2d**[d] | 4-ClPh/Me | >128 | 1 | 0.5 |

[a] Unit: μg/mL; [b] ref:[17]; [c] ref:[15]; [d] ref:[19].

At the meantime, several members of antibacterial CAAs also show significant anticancer activity ($IC_{50}$ in low μg/mL), which raises the concern that the antibacterial CAAs might be too toxic to mammalian cells (Table 2) [19-21]. Therefore, we further investigated the cytotoxicity of CAAs bearing alkyl groups at *N*-1 and *N*-3 positions and those with aryl groups at *N*-1 positions. The results show that these CAAs manifest various degree of cytotoxicity upon changing the substituents at the *N*-1 and *N*-3 positions. Furthermore, the structure of substituents appears to have profound effect on the cytotoxicity of normal and cancerous mammalian cells. For example, CAAs with only alkyl substituents (entries 1-3) show relatively low selectivity between normal (BEAS-2B) and cancerous (A549) mammalian cells. However, with the introduction of aromatic scaffold (entries 4-8), increasing toxicity toward cancerous cells was observed (indicated as increased selectivity). Additionally, the electronic effect of the substituents of the aromatic scaffold (entries 5-7) and the alkyl groups at *N*-3 (entries 7 vs. 8) seems to perturb the selectivity. CAA with electron-donating group, **2b** (OMe, entry 5) has similar toxicity as CAA with electron-withdrawing group, **2d** (Cl, entry 7) toward normal mammalian cell. However, **2b** is more toxic than **2d** toward cancerous cell.

Table 2. Cytotoxicity ($IC_{50}$) of CAAs[a]




| Entry | Compound | R/R′ | BEAS-2B (CCL-222) (lungs normal cell | A549 (CCL-185) (lungs cancerous cell) | Selectivity[b] |
|---|---|---|---|---|---|
| 1 | **1a** | Me/Me | 0.62±0.05 | 0.49±0.05 | 1.27 |
| 2 | **1b** | $C_8H_{17}$/Me | 0.60±0.02 | 0.43±0.03 | 1.40 |
| 3 | **1c** | $C_{16}H_{33}$/Me | 0.22±0.05 | 0.61±0.01 | 0.36 |
| 4 | **2a** | Bn/Me | 2.32±1.20 | 0.45±0.06 | 5.16 |
| 5 | **2b** | 4-MeOPh/Me | 5.80 ± 2.03 | 0.78 ± 0.16 | 7.44 |
| 6 | **2c** | Ph/Me | 5.76 ± 0.40 | 1.00±0.15 | 5.77 |
| 7 | **2d** | 4-ClPh/Me | 6.43±2.44 | 2.95±1.07 | 2.18 |

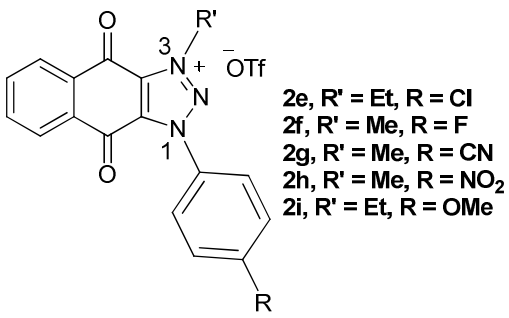
[a] Unit: μg/mL; [b] selectivity is calculated as $IC_{50}$(normal cell)/$IC_{50}$(cancer cell).

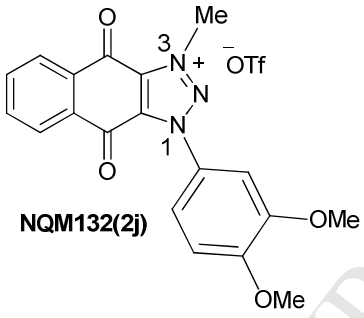
Combining the results from the antibacterial and cytotoxicity studies, we decided to investigate the electronic effect from the substituents of the aromatic scaffold at *N*-1 and the alkyl groups at *N*-3. Our goal is to identify new CAAs that are active specifically or selectively toward pathogenic *S. aureus*, not G- *E. coli* while maintaining low toxicity toward normal mammalian cells. To accomplish this goal, we opted to synthesize a library of new CAAs with variation on the substituents of the aromatic scaffold at *N*-1 first. Following the identification of the lead(s), more variation on the chain length of the alkyl group at *N*-3 will be commenced.

**Synthesis, Result and discussion**

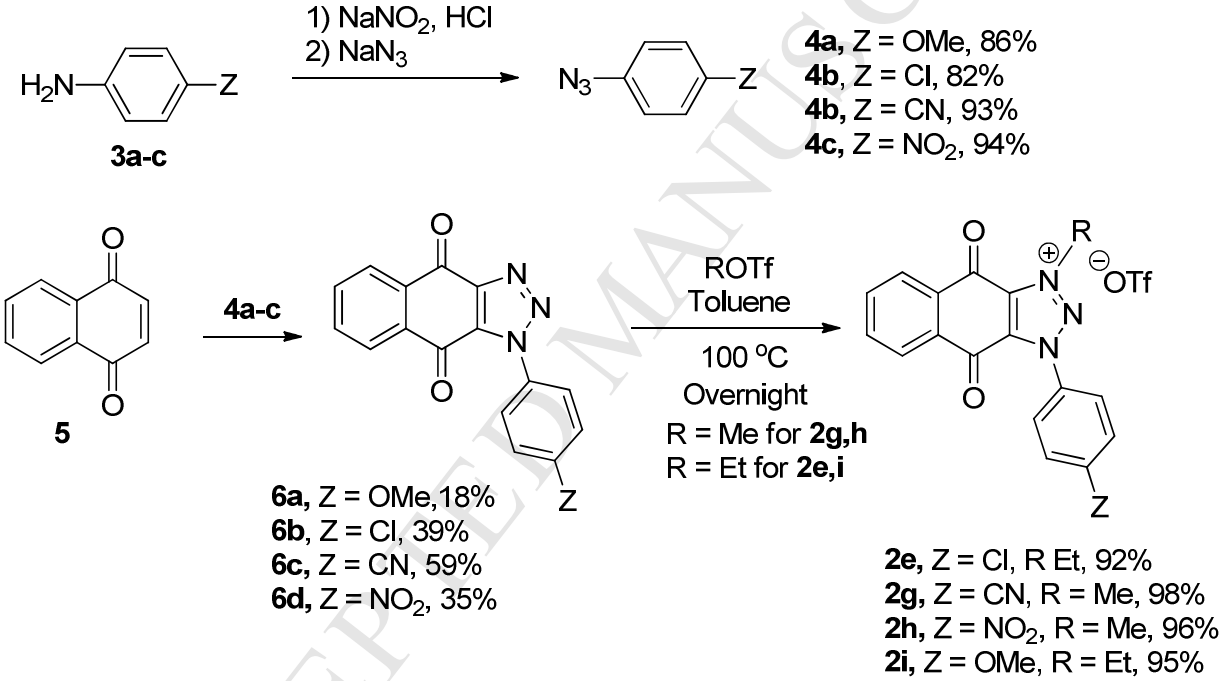
Six additional CAAs were selected or synthesized for further examination (Figure 1). Compounds **2f**, **2g** and **2h** have moderate to strong electron-withdrawing groups on the aromatic scaffold. Compound **2e** and **2i** have ethyl group at *N*-3 and compound **2j** has an extra methoxy group. Minimum inhibitory concentration (MIC) of these compounds towards MRSA can be compared with **2c**. The synthesis of compounds **2f** and **2j** has been reported previously [19]. Compounds **2e**, **2g**, **2h** and **2i** were synthesized following the reported protocol (Scheme 1).

Figure 1. CAAs for examining the effect of substituents of the aromatic scaffold



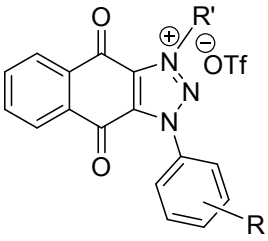




Scheme 1.




The antibacterial activity of the newly selected/synthesized CAAs summarized in Table 3. Additional strain of MRSA (ATCC43300) was also tested. All of these new members show comparable activity as the parent compounds, **2b** and **2c**, and are inactive (MICs ≥ 128 μg/mL) against *E. coli*. It is worth of mentioning that vancomycin is much less active against MRSA (ATCC43300) while all of the tested CAAs still maintain the same level of antibacterial activity. The high MIC of vancomycin against MRSA (ATCC43300) makes this strain of *S. aureus* actually falls into the category of VRSA or vancomycin intermediate (resistant) *S. aureus* (VISA).

Table 3. Antibacterial activity (MIC) of CAAs[a]




| Compound | R/R′ | *E. coli* (ATCC25922) | *S. aureus* (ATCC25923) | MRSA (ATCC 33591) | MRSA (ATCC 43300) |
|---|---|---|---|---|---|
| **2b** | 4-OMe/Me | >128 | 1-2 | 0.5 | 2 |
| **2c** | H/H | 128 | 1 | 0.5 | 2 |
| **2e** | 4 ClPh/Et | >128 | 2-4 | 1 | 2 |
| **2f** | 4-F/Me | >128 | 1 | 0.5 | 1 |
| **2g** | 4-CN/Me | >128 | 2 | 1 | 2 |
| **2h** | 4-$NO_2$/Me | >128 | 2 | 1 | 2 |
| **2i** | 4-OMe/Et | 128 | 4-8 | 2 | 2 |
| **2j** | 3,4-dimethoxy | >128 | 4 | 2 | 4 |
| Neomycin | - | 2 | 2 - 4 | 256 | > 256 |
| Vancomycin | - | > 256 | 2 | 2 | 32 |

[a] Unit: μg/mL

In addition of the BEAS-2B (CCL-222) (lungs normal cell)/A549 (CCL-185) (lungs cancer cell) pair, CCD-841-CoN (CRL-1790) (colon normal cells)/Colo205 (CCL-222) (colon cancer cells) pair of mammalian cells were also used for the cytotoxicity evaluation. Compound **2i** that has ethyl group at *N*-3 displays similar toxicity toward normal cells and toward cancerous cells resulting in similar selectivity but the antibacterial activity dropped. Despite similar activity of **2e**, ethyl at *N*-3, towards MRSA it is more toxic towards mammalian cells than methyl analog, **2d**. CAA with additional methoxy group exert increased toxicity toward normal and reduced selectivity. In contrast, CAAs with electron-withdrawing group(s) on the aromatic scaffold become less toxic toward normal cells. However, the decrease in toxicity toward cancerous cells was not as significant resulting in the increase in selectivity. Considering the selectivity and the cytotoxicity toward normal cells, compound **2h** was chose as the lead for investigating the effect of the alkyl chain length at *N*-3.

Table 4. Cytotoxicity ($IC_{50}$) of CAAs[a]

| Compound | BEAS-2B (CCL-222) (lungs normal cell | A549 (Lungs Cancer Cell) | Selectivity[b] | CCD-841-CoN (CRL-1790) | Colo205 (CCL-222) (colon | Selectivity[b] |
|---|---|---|---|---|---|---|

| | | | | (colon normal cells) | cancer cells) | |
|---|---|---|---|---|---|---|
| **2b** | 5.80 ± 2.03 | 0.78 ± 0.16 | 7.44 | 5.89±0.81 | 0.70±0.02 | 8.41 |
| **2c** | 5.76 ± 0.40 | 1.00±0.15 | 5.77 | 8.10 ± 0.80 | 0.90 ± 0.05 | 9.00 |
| **2e** | 4.22 ± 0.94 | 0.85 ± 0.06 | 4.96 | 6.65±1.342 | 0.78 ± 0.06 | 8.53 |
| **2j** | 3.76±0.29 | 1.60±0.55 | 2.35 | ND | ND | - |
| **2f** | 14.23±1.86 | 2.22±1.10 | 6.44 | 8.16 ± 0.23 | 2.86±0.38 | 2.85 |
| **2g** | 30.15±4.55 | 5.20±0.46 | 2.37 | 32.65±0.35 | 5.91± 0.28 | 5.52 |
| **2h** | 52.86±6.82 | 3.50±1.07 | 15.09 | 55.75±5.123 | 4.64± 0.25 | 11.99 |
| **2i** | 5.00 ± 0.22 | 0.73 ± 0.08 | 6.85 | 7.85± 1.04 | 0.82± 0.06 | 9.57 |

[a] Unit: μg/mL; [b] selectivity is calculated as $IC_{50}$(normal cell)/$IC_{50}$(cancer cell).

Using **2h** as the lead, the ethyl, butyl, hexyl, octyl and decyl groups were incorporated at the *N*-3 position (Scheme 2). These compounds were tested again for their activity against *S. aureus* and toxicity (Tables 5 and 6). Overall antibacterial activity of these analogs with extended alkyl chain were mostly inferior to the parent compound, **2h**. In general, the antibacterial activity decreases with decreasing chain length. There is an interesting structure-activity relationship (SAR) between the cytotoxicity and chain length. Except **2h**, the cytotoxicity decreased (increased $IC_{50}$) initially as the chain length increased, reached minimum with butyl or hexyl groups and increased (decreased $IC_{50}$) as the chain length further increased. Same SARs were observed for all of the examined cell lines (normal and cancerous). Overall, the parent **2h** still has the lowest toxicity toward normal cells and best antibacterial activity against *S. aureus*

Scheme 2.

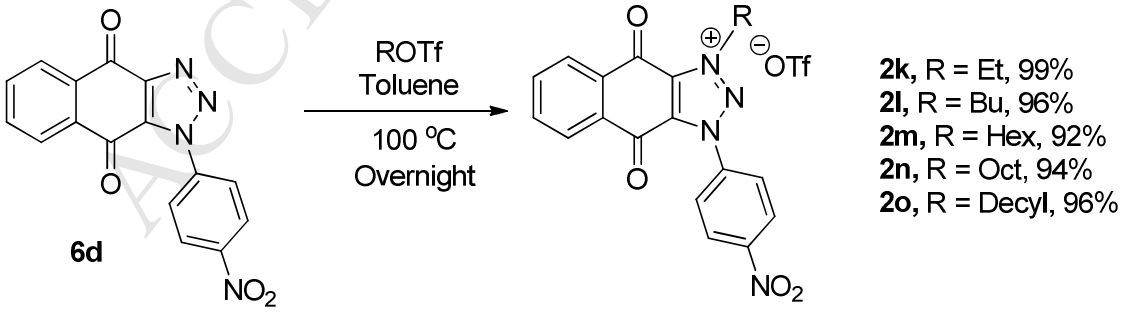



Table 5. Antibacterial activity (MIC) of CAAs[a]

| Compound | R | *S. aureus* | MRSA | MRSA |
|---|---|---|---|---|

| | | (ATCC25923) | (ATCC 33591) | (ATCC 43300) |
|---|---|---|---|---|
| **2k** | ethyl | 8-16 | 1-2 | 4-8 |
| **2l** | butyl | 4 | 2 | 8-16 |
| **2m** | hexyl | 2-4 | 1 | 128 |
| **2n** | octyl | 8 | 16 | 32 |
| **2o** | decyl | 2-4 | 8 | 8-16 |

[a] Unit: μg/mL

Table 6. Cytotoxicity ($IC_{50}$) of CAAs[a]

| Compound | BEAS-2B (CCL-222) (lungs normal cell | A549 (Lungs Cancer Cell) | Selectivity[b] | CCD-841-CoN (CRL-1790) (colon normal cells) | Colo205 (CCL-222) (colon cancer cells) | Selectivity[b] |
|---|---|---|---|---|---|---|
| **2k** | 43.56±2.67 | 7.40±0.28 | 5.89 | 51.26±5.86 | 7.72±0.40 | 6.64 |
| **2l** | 49.75±5.73 | 44.89±5.17 | 1.11 | 42.79 ± 1.48 | 43.17 ± 4.53 | 0.99 |
| **2m** | 38.58±0.81 | 48.88±2.66 | 0.79 | 40.19 ± 7.18 | 21.25 ± 3.24 | 1.89 |
| **2n** | 20.98 ± 2.37 | 15.59 ± 4.411 | 4.76 | 30.98 ± 3.57 | 8.53 ± 0.71 | 3.63 |
| **2o** | 7.39 ± 0.65 | 7.02 ± 2.16 | 1.05 | 30.64 ± 5.30 | 7.29 ± 0.24 | 4.20 |

[a] Unit: μg/mL; [b] selectivity is calculated as $IC_{50}$(normal cell)/$IC_{50}$(cancer cell).

Following these SAR investigation, the electronic effect from the substituents of the aromatic scaffold at *N*-1 and the effect of alkyl groups at *N*-3 on the antibacterial activity and cytotoxicity are illustrated in Figures 2 and 3. It is clear that having electron-withdrawing groups (ex. $NO_2$ and CN) on the aromatic scaffold at *N*-1produces CAAs with superior antibacterial activity and *selective* cytotoxicity toward cancerous cells (Figure 2). Such selectivity between antibacterial and anticancer, and normal and cancerous mammalian cells diminishes as weaker electron-withdrawing group (Cl) or electron-donating group (OMe) are attached. With few exceptions, the general trend regarding the SAR of varying chain-length at *N*-3 is that short alkyl chain (Me) is better than longer alkyl chains (Et, butyl, hexyl and octyl) (Figure 3).

Figure 2. Electronic effect from the substituents of the aromatic scaffold

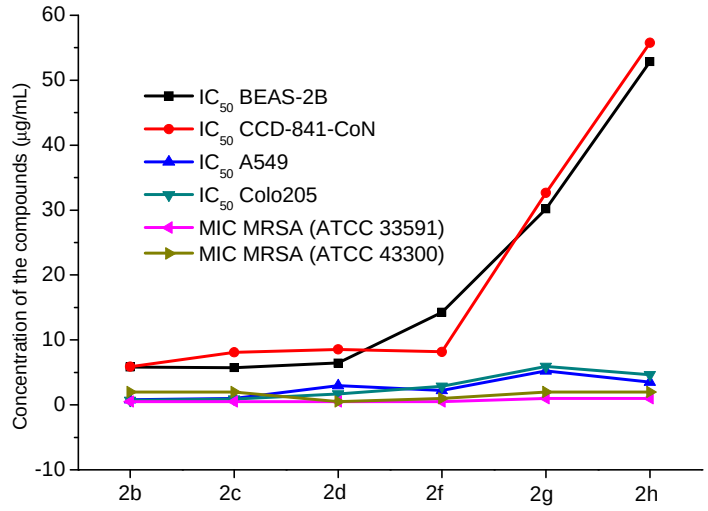
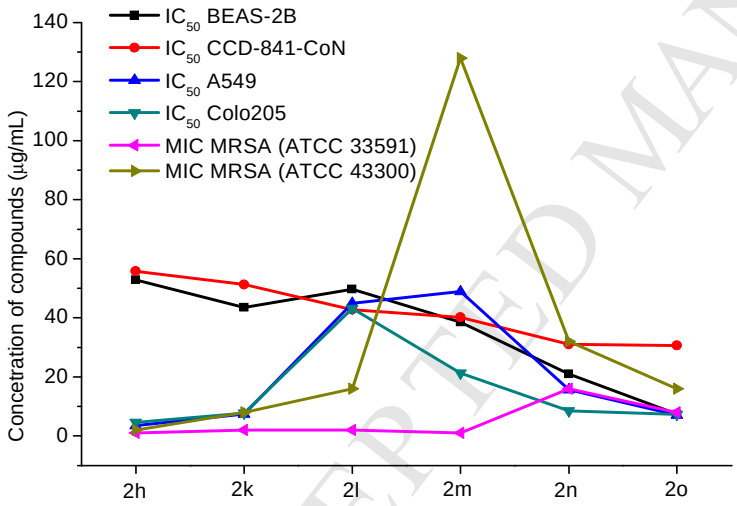



Figure 3. Effect of variation of alkyl groups at *N*-3

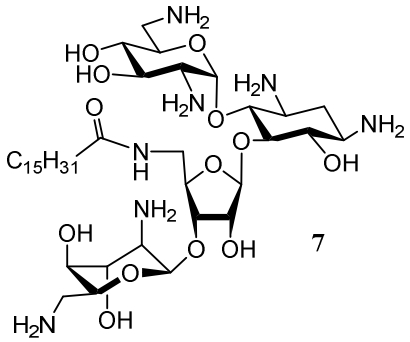



In general, most of the reported CAAs possess significant antibacterial activity against *S. aureus.* These CAAs are more active than previously reported quinone based antibacterial compounds like phaeosphenone, Lawson and Lawson derivatives [22-24] and have similar activity like naphthazarins [25]. We have previously reported that the anticancer CAAs exert the activity by inducing the production of reactive oxygen species (ROSs) leading to the damage of mitochondria membrane, a site that is not readily accessible as bacteria membrane [21]. In addition, we have also noticed that the attachment of electron-withdrawing group on the aryl group of CAAs can lower the cytotoxicity due to the perturbation of redox potential. We have discovered that cancerous cells are more prone toward the oxidative stress (high ROS level) induced by CAAs as compared to normal cells. Combining these finding, it provides the

rationale for supporting the reported observation. First, the redox active CAAs exert antibacterial activity by disrupting the redox process of bacteria via, likely, the production of ROSs: a common MOA that has been noticed in other quinone-based antibacterial compounds[26]. Second, since cancerous cells are prone to oxidative stress, the presence of nitro group makes compound **2h** less toxic toward normal mammalian cells than cancerous cells. Third, the sites targeted by CAAs for the antibacterial and anticancer MOAs are different (bacterial vs. mitochondrial membranes). Therefore, it is possible that the easy accessibility of bacterial membrane leads to the observation that the electronic effect of the substituents on CAAs does not influence their antibacterial activity as much as their anticancer activity.

To augment the application of the lead compound, **2h**, the antibacterial activity using combination of **2h** with commercially used antibacterial compounds fosfomycin and ciprofloxacin, and previously reported membrane disrupting antibacterial compounds **7** (Figure 4) [27] were studied. Result showed that **2h** does not have any synergistic effect with the tested compounds.

Figure 4. Structure of **7**

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**Conclusion-**

CAAs are generally highly active against various strains of G+ bacterial pathogens, including MRSA. Most of the CAAs are much less active against G- bacteria, an advantage that can be utilized for the development of selective antibiotic and avoid CDI. However, several members of CAAs also exert moderate to significant cytotoxicity toward mammalian cells. It has been noted that the substituents at the *N*-1 and *N*-3 positions of CAAs play significant role in the observed biological activity. In order to tune the activity of CAAs toward MRSA while maintaining low cytotoxicity toward mammalian cells, a library of CAAs was synthesized and assayed to reveal the SAR. Results from cytotoxicity antibacterial assay showed that *N*-1 and *N*-3 alkyled CAAs have either no selectivity or very poor selectivity between G+ bacteria and mammalian cells (normal and cancerous). Nevertheless, when attaching aryl motif with electron-withdrawing groups at *N*-1 position, CAAs become much less toxic toward mammalian cells while still maintaining the antibacterial activity. Further screening the CAAs with various electron-

withdrawing groups against normal and cancerous cells allows the identification of **2h** equipped with $NO_2$ as the lead compound. SAR based on the variation of alky chain length revealed that methyl group is the optimal structural motif. Compound **2h** stands out with the best selectivity between bacteria vs. mammalian cells, and normal mammalian cells vs. cancerous cells. The discovery of **2h** may pave the way toward the development of new antibacterial agent against MRSA and VRSA.

**Experimental section**

**General procedure A for the synthesis of 2e, 2i, 2k:** Ethyl triflate used on the synthesis of these compounds was synthesized from the reaction of ethanol with triflic anhydride using pyridine as the base. 8 equivalents of triflic anhydride was added dropwise to the stirred solution of 1 equivalent of ethanol and 8 equivalents of the pyridine in the anhydrous toluene maintaining the temperature below 0 $^{o}$C. Reaction mixture was stirred for 2 hours and filtered through the anhydrous sodium sulfate. Ethyl triflate in filtrate was added to the 1 equivalent of the **6a**, **6b**, or **6d** and refluxed at 100 $^{o}$C overnight. Toluene from reaction mixture was remove under reduced pressure and the residue washed with the diethyl ether to obtain pure products.

**General procedure B for the synthesis of 2l–2o:** When pyridine was used as the base to synthesize these compounds as the general procedure A using alcohols with different chain length, less than 5% of the expected products were obtained. The side products of those reaction were the alkyl substituted pyridine. To avoid the alkylation of the pyridine, less nucleophilic base triethylamine was used. 8 equivalents of triflic anhydride was added dropwise to the stirred solution of 1 equivalent of alkyl alcohol and 8 equivalents of triethylamine in anhydrous toluene over the thirty minutes keeping the temperature below 0 $^{o}$C. Then the reaction keeps stirred for another 2 hours. To the reaction flasks **6d** was added and refluxed at 100 $^{o}$C overnight. Next day reaction was cooled to room temperature and toluene removed under reduced pressure. Thus, obtained residue washed with water and diethyl ether to obtain final products.

**General procedure for the antibacterial activity-** Minimum inhibitory concentration of the compounds were tested against *E. coli* (ATCC25922), *S. aureus* (ATCC25923), *S. aureus* MRSA (ATCC 33591), and *S. aureus* MRSA (ATCC 43300) as the reported procedure[28].

**General procedure for the cytotoxicity-** Cytotoxicity of the compounds was evaluated by colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye**.** A549 (CCL-185), BEAS-2B (CCL-222), Colo205 (CCL-222), and CCD-841-CoN (CRL-1790) cells were used to assess the cytotoxicity of the compounds. A549 and BEAS-2B were grown in the Dulbecco's modification of eagle's medium (DMEM), (Corning) supplemented with 10 percent fetal bovine serum (FBS) (Hyclone, SH3007103). Similarly, Colo205 cells were grown in RPMI1640 (ATCC 302001) and CCD-841-CoN cells in Eagle's minimum essential medium (EMEM) (ATCC 302003) supplemented with 10% FBS. 100 U/mL of streptomycin and 100 U/mL of penicillin were treated to all these culture media.

200 uL of the growing medium containing enough cells to get sub confluent culture and good absorption value of at the end of the assay, 5000 cells for A549 and BEAS-2B cells and 15,000 cells for colo205 and CCD-841-CoN, were added to the 96 wells cell culture plate (Corning). After 24 hours media were removed and the different concentration of drugs (0.1, 1, 10, 100 µg/mL) diluted in culture medium were added and incubated for 48 hours in the incubator at 37 $^{o}$C with 5 % $CO_2$ environment. Then 20 uL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye (5 mg/mL) was added and incubated in the same condition. After 5 hours, media was removed, and the reduced formazan dye was dissolved in DMSO. Absorption of the dissolved formazan was taken at 570 nm with 650 nm as background in micro titer plate reader (BioTek). Relative viability of the cells was calculated by comparing the absorption of the compound treated cells to control cells, which is considered to have 100% cell viability.

**Material and Methods**

Solvents and reagents were purchased from the commercial source. Alcohols used in the synthesis of the alkyltriflate was dried over molecular sieve for 48 and toluene was dried using calcium hydride. $^{1}$H NMR and $^{13}$C NMR spectra were taken in Bruker Ascend 500 under 500 MHZ and 75 MHZ respectively. Melting point of the compounds were recorded using DigiMelt MPA160.

**4-(4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-1-yl)benzonitrile (6c)-** This compound is synthesized as discribed[19] by the cyclization of naphthoquinone with aryl azide with 60 % yield. **6c** is yellowish white solid with melting point more than 260$^{o}$C. $^{1}$H NMR (500 MHz, DMSO-$d_6$) δ 8.26 (dd, *J* = 9.0 Hz, *J* = 1.5 Hz, 1H), 8.21 (d, *J* = 8.5 Hz, 2H) 8.15 (dd, *J* = 7.5 Hz, *J* = 1.5 Hz, 1H) 8.07 (d, *J* = 8.5 Hz, 2H) 7.9 – 8.0 (m, 2H)); $^{13}$C NMR (125 MHz, DMSO-d6) δ 177.46, 174.39, 145.47, 139.00, 135.62, 135.49, 135.31, 134.01(2C), 133.73, 133.12, 127.58, 127.42, 126.90 (2C), 118.42, 113.81. ESI/APCI calculated for [$C_{17}H_9N_4O_2^+$]: 301.0726; Measured m/e: 301.0725.

**1-(4-cyanophenyl)-3-methyl-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2g) – 2g** is synthesized by the reaction of compound **6c** with methyltriflate[19] with 98 % yield. This compound is light grey solid with melting point 240-241 $^{o}$C. $^{1}$H NMR (500 MHz, DMSO- $d_6$) δ 8.3 – 8.4 (m, 3H), 8.2 – 8.3 (m, 3H), 8.1 – 8.2 (m, 2H), 4.84 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-d6) δ 173.02, 171.47, 137.15, 136.89, 136.80, 136.66, 135.99, 134.71 (2C), 133.08, 132.17, 128.51, 128.31, 127.40 (2C), 117.89, 116.22, 41.69.ESI/APCI calculated for[$C_{18}H_{11}N_4O_2^+$]: 315.0877; measured m/e: 315.0866.

**3-methyl-1-(4-nitrophenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2h)-** This is synthesized by following the same procedure as compound **2g** with 96 % yield. **2h** is grey solid with melting point 248-249 $^{o}$C. $^{1}$H NMR (500 MHz, Methanol- $d_3$) δ 8.65 (d, *J* = 9.0 Hz, 2H), 8.4 – 8.5 (m, 1H), 8.3 – 8.4 (m, 1H), 8.24 (d, *J* = 9.0 Hz, 2H) 8.1 – 8.2 (m, 2H), 4.89 (s, 3H); $^{13}$C NMR (125 MHz, Methanol-D3) δ 172.34, 170.91, 150.44, 137.62, 136.18, 136.14 (2C), 136.02, 132.73, 132.14, 127.98, 127.74, 127.40

(2C), 124.81 (2C), 40.42. ESI/APCI calculated for [$C_{17}H_{11}N_4O_4^+$ + MeOH]: 367.1037; Measured m/e: 367.1113.

**3-ethyl-1-(4-methoxyphenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2i)- C**ompound is synthesized by following the general procedure A with 95 % yield. **2i** is yellow solid with melting point 155-156 $^oC$. $^1H$ NMR (500 MHz, Methanol- $d_3$) δ 8.4 -8.5 (m, 1H), 8.3 – 8.4 (m, 1H), 8.0 – 8.1 (m, 2H), 7.8-7.9 (m, 2H) 7.2 – 7.3 (m, 2H), 5.25 (q, *J* = 7.0 Hz, 2H), 1.83 (t, *J* = 7.0 Hz, 3H). $^{13}C$ NMR (125 MHz, Methanol-D3) δ 172.71, 171.14, 163.17, 135.92, 135.69, 132.82, 132.26, 127.82, 127.57, 127.06 (2C), 126.27, 114.48 (2C), 55.16, 50.43, 12.77. ESI/APCI calculated for[$C_{19}H_{16}N_3O_3^+$]: 334.1186; Measured m/e:- 334.1205.

**1-(4-chlorophenyl)-3-ethyl-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2e)-** This compound is synthesized by following the general procedure A with 92 %Yield. **2e** is greenish white solid with melting point 197-198 $^oC$. $^1H$ NMR (500 MHz, Methanol- $d_3$) δ 8.4 – 8.5 (m, 2H), 8.3 – 8.54 (m, 1H), 8.0 – 8.1 (m, 2H), 7.95 (dt, *J* = 9.0 Hz, *J* = 3.0 Hz, 2H) 7.83 (dt, *J* = 9.0 Hz, *J* = 3.0 Hz, 2H) 5.28 (q, *J* = 7.0 Hz 2H), 1.84 (t, *J* = 7.0 Hz 3H); $^{13}C$ NMR (125 MHz, Methanol-D3) δ 172.48, 171.07, 139.05, 136.04, 135.89, 135.76, 132.25, 130.22, 129.79 (3C), 127.90, 127.70, 127.24 (2C), 123.13, 50.76, 12.75. ESI/APCI calculated for338.0691; Measured m/e: 338.0687.

**3-ethyl-1-(4-nitrophenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2k):** Compound **2k** is synthesized by following the general procedure A with the 0.331 gm 99 % yield. This compound is grey in color with melting point 203-205 $^oC$. $^1H$ NMR (500 MHz, Methanol- $d_3$) δ 8.6 – 8.7 (m, 2H), 8.4 – 8.5 (m, 1H), 8.3-8.4 (m, 1H) 8.2 – 8.3 (m, 2H), 8.0 – 8.1 (m, 2H), 5.33 (q, *J* = 7.5 Hz, 2H), 1.86 (t, *J* = 7.5 Hz, 3H). $^{13}C$ NMR (125 MHz, Methanol-D3) δ 172.31, 171.04, 150.44, 137.73, 136.37, 136.13, 136.06, 135.85, 132.59, 132.24, 127.97, 127.80, 127.42 (2C), 124.80 (2C), 51.07, 12.74. ESI/APCI calculated for [$C_{19}H_{16}N_3O_3^+$]: 334.1186; Measured m/e: 334.1205.

**3-butyl-1-(4-nitrophenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2l)-** Compounds is synthesized by following the general procedure B with the 0.504 gm (95.81 %) yield. This compound is grey in color with melting point 199-200 $^oC$. $^1H$ NMR (500 MHz, Methanol- $d_3$) δ 8.6 -8.7 (m, 2H), 8.4 – 8.5 (m, 1H), 8.3-8.4 (m, 1H) 8.2 – 8.3 (m, 2H), 8.0 – 8.1 (m, 2H), 5.29 (t, *J* = 7.5 Hz, 2H), 2.2 – 2.3 (m, 2H), 1.6 – 1.7 (m, 2H), 1.10 (t, *J* = 7.0 Hz, 3H). $^{13}C$ NMR (125 MHz, Methanol-D3) δ 172.33, 170.99, 150.46, 137.66, 136.41, 136.18, 136.09, 135.85, 132.58, 132.24, 127.99, 127.83, 127.40 (2C), 124.82 (2C), 54.85, 30.58, 19.12, 12.27. ESI/APCI calculated for [$C_{20}H_{17}N_4O_4^+$]: 377.1244; Measured m/e: 377.1214.

**3-hexyl-1-(4-nitrophenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2m)-** Compounds is synthesized by following the general procedure B with the 0.509 gm (91.87 %) yield. This compound is grey in color with melting point 195-196 $^oC$. $^1H$ NMR (500 MHz, Methanol- $d_3$) δ 8.67 (d, *J* = 9.0 Hz, 2H), 8.4 – 8.5 (m, 1H), 8.3-8.4 (m, 1H) 8.25 (d, *J* = 9.0 Hz, 2H), 8.0 – 8.1 (m, 2H), 5.28 (t, *J* = 7.5 Hz, 2H), 2.2 –

2.3 (m, 2H), 1.5 – 1.6 (m, 2H), 1.3 - 1.5 (m, 4H), 0.97 (t, *J* = 7.0 Hz, 3H). $^{13}$C NMR (125 MHz, Methanol-D3) δ 172.33, 171.02, 150.44, 137.68, 136.41, 136.41, 136.15, 136.09, 135.85, 132.59, 132.25, 127.97, 127.82, 127.42 (2C), 124.81 (2C), 55.07, 30.79, 28.62, 25.55, 22.03, 12.27. ESI/APCI calculated for [$C_{22}H_{21}N_4O_4^+$]: 405.1557; Measured m/e: 405.1583.

**1-(4-nitrophenyl)-3-octyl-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2n)-** Compounds is synthesized by following the general procedure B with the 0.546 gm (93.97 %) yield. This compound is grey in color with melting point 92-93 $^{o}$C. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.32 (d, *J* = 9.0 Hz, 2H), 8.2 – 8.3 (m, 1H), 8.1-8.2 (m, 1H) 7.8 - 8.0 (m, 4H), 8.0 – 8.1 (m, 2H), 4.85 (t, *J* = 7.5 Hz, 2H), 2.0 - 2.1 (m, 2H), 1.2 – 1.5 (m, 10H), 0.90 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 172.27, 171.16, 149.78, 137.92, 136.25, 135.69, 135.62(2C), 132.42, 132.19, 128.17, 127.99, 127.91, 124.55 (2C), 123.74 (2C), 54.96, 31.67, 28.91, 28.72, 28.29, 26.29, 25.57, 22.57, 14.05. ESI/APCI calculated for [$C_{24}H_{25}N_4O_4^+$]: 433.1870; Measured m/e: 433.1900.

**3-decyl-1-(4-nitrophenyl)-4,9-dioxo-4,9-dihydro-1*H*-naphtho[2,3-*d*][1,2,3]triazol-3-ium trifluoromethanesulfonate (2o)-** Compound is synthesized by following the general procedure B with the 96 % yield. **2o** is brown solid with melting point 82-83 $^{o}$C. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.3 – 8.4 (m, 2H), 8.2 – 8.3 (m, 1H), 8.1-8.2 (m, 1H) 7.8 - 8.0 (m, 4H), 4.85 (t, *J* = 7.5 Hz, 2H), 2.0 - 2.1 (m, 2H), 1.2 – 1.5 (m, 14H), 0.89 (t, *J* = 7.5 Hz, 3H). $^{13}$C NMR (125 MHz, $CDCl_3$) δ 172.26, 171.16, 149.79, 137.92, 136.25, 135.69, 135.63 (2C), 132.43, 132.19, 128.19, 128.00, 127.91 (2C), 124.56 (2C), 54.97, 31.84, 29.45, 29.27, 29.23, 28.91, 28.77, 26.30, 22.66, 14.10. ESI/APCI calculated for [$C_{26}H_{29}N_4O_4^+$]: 461.2183; Measured m/e: 461.2158.

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## Highlights

1. Substituent at *N*-3 can tune the selectively of compound toward G+ bacteria.
2. Compound **2h** have antibacterial activity against MRSA but not G- bacteria.
3. Compound **2h** is less toxic towards normal mammalian cells compared to cancerous cells.