Synthesis and Anti-proliferative Activity of *N*,*N*′-bis-substituted 1,2,4-Triazolium Salts against Breast Cancer and Prostate Cancer Cell Lines

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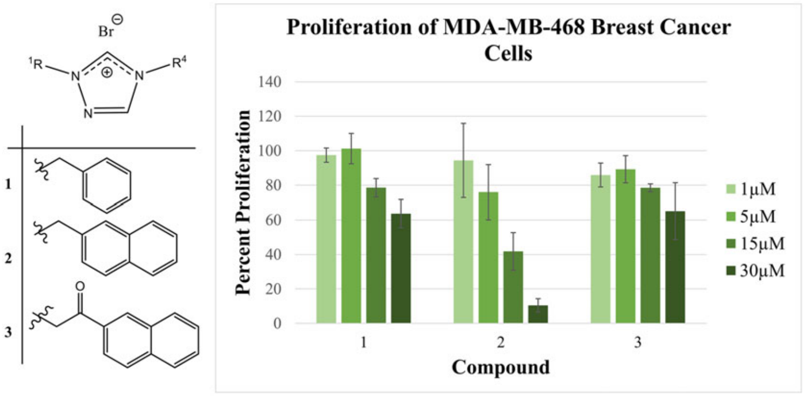

A series of 1,4-*N*,*N*′-bis-substituted 1,2,4-triazolium bromide salts were synthesized and tested for anti-proliferative activity. 1,4-Bis(naphthalen-2-ylmethyl)-1,2,4-triazolium bromide (**2**) showed activity against MDA-MB-468 breast cancer and PC-3 prostate cancer cell lines.

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INTRODUCTION

The continued challenge of combating cancer, America's second leading cause of death, has driven wide-ranging exploration into novel anticancer therapies [1]. Imidazolium and benzimidazolium salts have recently been shown to have anticancer activity in non-small cell lung cancer cell lines [2–4]. Recent advances in the design of these nitrogen-containing heterocyclic compounds, such as imidazolium and benzimidazolium salts, have highlighted the importance of substituent properties, particularly hydrophilicity, on therapeutic potential [2,3]. Hydrophilicity is critical to drug absorption and distribution in aqueous biological solutions (e.g., blood). There is a constant effort to balance drug activity and bioavailability. This holds true for imidazolium and benzimidazolium salts, which despite potent anticancer activity, exhibit poor aqueous solubility. Considering these challenges, triazolium salts are attractive targets for optimization as effective antitumor agents because of their increased dipole moment.

Triazole derivatives have been used extensively for medicinal purposes ranging from analgesic to anti-inflammatory properties [5,6]. Additionally, progress has been made in the design of triazole-derived antimicrobial compounds [6]. One of these compounds, 1,4-dibenzyl-1,2,4-triazolium bromide (**1**, Fig. 1), has been shown to be an antimalarial agent; however, its anti-proliferative effect on cancer cells has not been reported [7,8].

Two of the most common cancers found in men (prostate) and women (breast) generally start as hormone-dependent tumors [9,10]. However, some breast and prostate cancers are hormone independent [11]. The goal of this study is to investigate substituent properties, particularly hydrophilicity, on the therapeutic potential of 1,2,4-triazole derivatives in hormone-independent breast and prostate cancers. Additionally, the effect of the heterocycle parent was explored for three bis(naphthylmethyl)-substituted heterocyclic bromide salts.

Salt **1** was synthesized and tested for anticancer activity against hormone-independent breast and prostate cancer cell lines (MDA-MB-468 and PC-3, respectively). Two additional *N*,*N*′-bis-substituted 1,2,4-triazolium bromide salts (**2** and **3**, Fig. 1) were synthesized and tested to determine the effect of the solubilizing group on aqueous solubility and anticancer activity against these cell lines. The role of the parent heterocycle was also explored by comparing new compounds with literature imidazolium salts **4** and **5** (Fig. 1). All treatments were compared with the chemotherapeutic agent tamoxifen [11].

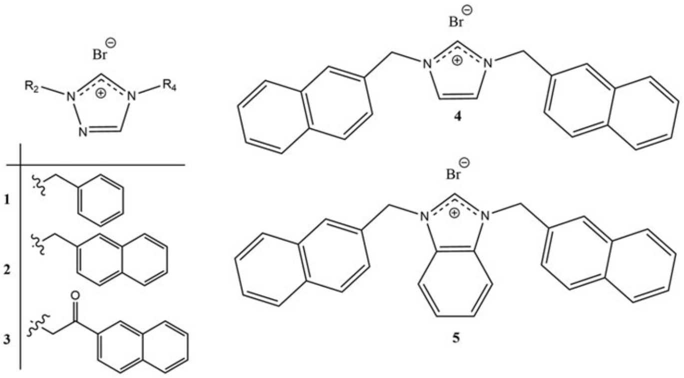
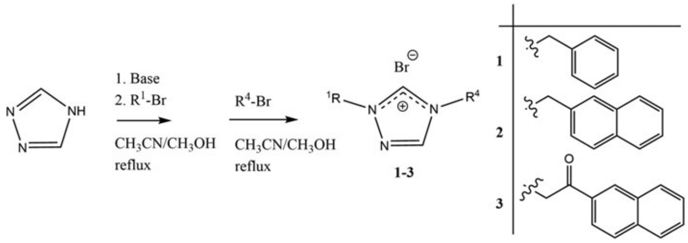


Figure 1. Schematic representation of *N*,*N′*-bis-substituted 1,2,4-triazolium bromide salts (**1–3**) and literature imidazolium bromide salts (**4** and **5**).

Scheme 1. Synthesis of the *N*,*N′*-bis-substituted-1,2,4-triazolium bromide salts (**1–3**).



RESULTS AND DISCUSSION

Scheme 1 outlines the synthetic procedure of the *N*,*N′*-bis-substituted 1,2,4-triazolium salts **1–3** from commercially available 1,2,4-triazole with yields ranging from 26 to 62%. The salts were prepared by stirring the 1,2,4-triazole with a minimum of 1.1 equivalents of a hydroxide base (NaOH or KOH) in acetonitrile and alcohol solvent mixture (9:1, v : v) and one molar equivalent of the alkyl bromide to yield the mono-substituted intermediate. Following the removal of the white precipitate, presumed to be a bromide salt (NaBr or KBr), a second equivalent of the alkyl halide was added. The mixture was heated at reflux overnight to yield **1–3**. Salt **3** was isolated in a low yield. The electron-withdrawing nature of the acyl group is likely to decrease the nucleophilicity of the mono-substituted 1,2,4-triazole intermediate and lowering the yield. Inclusion of the carbonyl group on the alkyl halide reagent may have also contributed to undesired side reactions. Salts **4** and **5** were synthesized according to literature procedures [2].

All *N*,*N′*-bis-substituted 1,2,4-triazolium bromide salts **1–3** were characterized by ^{1}H and ^{13}C NMR spectroscopy, high-resolution mass spectrometry (MS), elemental analysis, and melting point determination. ^{1}H NMR spectroscopy confirmed the formation of the *N*,*N′*-bis-substituted 1,2,4-triazolium bromide salts **1–3** with the downfield shift in the ^{1}H NMR of the C^5-H triazolium proton between 10 and 11 ppm. Similarly, alkylation of N^1 and N^4 nitrogen atoms in the triazole ring and transformation into the *N*,*N′*-bis-substituted 1,2,4-triazolium salts were corroborated by the ^{13}C NMR spectra. Electrospray ionization (ESI)-MS in the positive mode was conducted for **1–3**, and the corresponding [M-Br]$^+$ signal further confirmed identification of the salts. The structure of **2** was further confirmed by X-ray crystallography (Fig. 2). Single crystals were grown from the slow evaporation of a concentrated solution of ethanol. The carbonyl frequencies of **3** were observed at 1685 cm^{-1} (C=O) and 1702 cm^{-1} (C=O) in the infrared spectroscopy.

The antimicrobial activity and planar benzyl substituents of **1** made it an attractive candidate for potential anticancer activity. Compound **1** had favorable water solubility (log $P = -1.88 \pm 0.06$, Table 1) but exhibited poor anti-proliferative activity against the breast cancer cell line ($IC_{50} = 86.4 \pm 0.2$ μ*M*, Table 1) with more than 70%

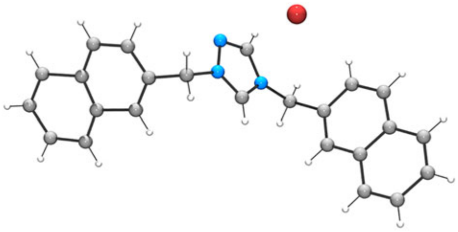


Figure 2. Ball and stick structure of **2** based on X-ray crystallography data. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1

IC_{50} values of compounds **1**–**5** and tamoxifen dissolved in aqueous growth media against breast cancer (MDA-MB-468) and prostate cancer (PC-3) cell lines.

Compound	Breast (μ*M*)	Prostate (μ*M*)	Log *P*
1	86.4 ± 0.2	20.14 ± 0.04	−1.88 ± 0.06
2	15.79 ± 0.37	–	0.17 ± 0.01
3	100 ± 0.15	–	0.6 ± 0.1
4	5.0 ± 0.4	3.4 ± 0.3	0.73 ± 0.08
5	9.7 ± 0.4	35.07 ± 0.36	1.3 ± 0.3
Tamoxifen	22.5 ± 0.4	9.58 ± 0.23	

The octanol/water partition coefficient is reported as the log *P* for compounds **1**–**5**. IC_{50} was not determined for **2** and **3** because of very low activity.

breast cell proliferation remaining at 30 μ*M* (Fig. 3). Compound **1** was fivefold more potent against the prostate cancer cell line (IC_{50} = 20.14 ± 0.04 μ*M*).

Previous work indicated that a bis(naphthylmethyl)-substituted imidazolium salt was more effective against tested cancer cells than the dibenzyl analogue [2]. Therefore, the bis(naphthylmethyl) triazolium salt **2** was synthesized and tested. Salt **2** displayed a substantial increase in potency (IC_{50} = 15.79 ± 0.37 μ*M*) and anti-proliferative activity with less than 15% proliferation at 30 μ*M* against the breast cancer cell line compared with **1**. Salt **2** was also more potent than tamoxifen (IC_{50} = 22.5 ± 0.4 μ*M*) against the breast cancer cell line. However, the increased activity came at the expense of solubility (log *P* = 0.17 ± 0.01). Salt **2** was much less effective against the prostate cancer cell line (75 ± 4% proliferation remaining at 30 μ*M*, Fig. 4). This apparent differential activity suggests that modification of the parent heterocycle may provide a route to cell selectivity.

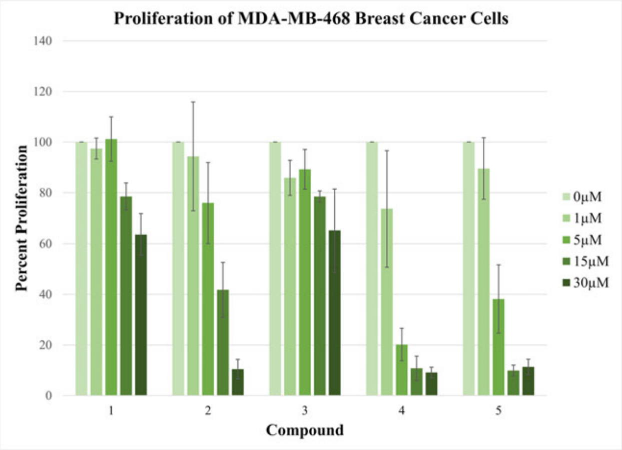


Figure 3. Proliferation of MDA-MB-468 breast cancer cells treated with **1**–**5**. Compounds **1** and **3** had very little anti-proliferative effects even at 30 μ*M*. Compounds **2**, **4**, and **5** showed similar anti-proliferative effects at 30 μ*M*. [Color figure can be viewed at wileyonlinelibrary.com]

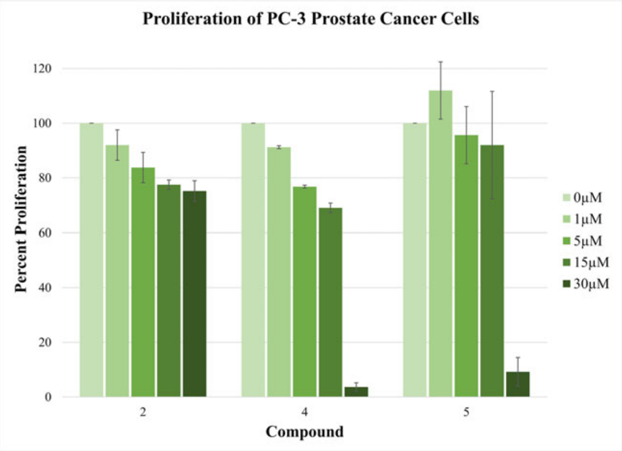


Figure 4. Proliferation of PC-3 prostate cancer cells treated with **2**, **4**, and **5**. Compounds **4** and **5** showed similar anti-proliferative effects against both cancer types. In contrast to activity against the breast cancer cell line, salt **2** exhibited very low activity against the prostate cell line. [Color figure can be viewed at wileyonlinelibrary.com]

The rigid ring structure of the naphthyl substituent was necessary for the activity of a bis(naphthylmethyl) imidazolium salt **4** and a bis(naphthylmethyl) benzimidazolium salt **5** against several non-small cell lung cancer cell lines [2]. However, the introduction of an unsubstituted heteroatom and hydrogen bond acceptor in the parent compound was not explored. The favorable solubility and activity of **2** warranted comparisons with reported salts **4** and **5**.

Introduction of the additional unsubstituted nitrogen increased water solubility of **2** by fourfold (log $P = 0.17 \pm 0.01$) compared with **4** (log $P = 0.73 \pm 0.08$, Table 1). Salt **5** was significantly less soluble (log $P = 1.3 \pm 0.3$, Table 1). Interestingly, while **2** exhibited increased aqueous solubility, potency was slightly decreased against the breast cancer line when compared with **4** and **5** (Table 1). However, the anti-proliferative activity of **2** was essentially unchanged at 30 μ*M* versus **4** and **5** (Fig. 1).

In an attempt to further increase the solubility of the *N*, *N′*-bis-substituted 1,2,4-triazolium salts, salt **3**, containing the naphthylacyl substituent, was synthesized and tested. Aqueous solubility of **3** was slightly decreased (log $P = 0.6 \pm 0.1$, Table 1) compared with **2**, and the biological activity was all but eliminated in both cell lines. The decrease in activity, caused by the introduction of the carbonyls near the parent heterocycle, warrants further investigation.

CONCLUSION

A set of *N*,*N′*-bis-substituted 1,2,4-triazolium salts with solubilizing groups at the N^1 and N^4 were synthesized. Results showed that salt **1** did not possess substantial anticancer activity, regardless of displaying antimalarial properties. Furthermore, the naphthylmethyl substituent was required for anticancer activity in both breast and prostate cell lines. However, the comparison of salts **2**, **4**, and **5** indicated that the 1,2,4-triazole heterocycle offered an increased aqueous solubility and overall hydrophilicity. Future work on azolium salts would include the investigation of other parent heterocycles, in addition to the exploration of other solubilizing groups on the atoms (N^2, C^3, and C^5) in the triazole ring.

EXPERIMENTAL

General considerations. 1,2,4-Triazole and 2-(bromoacetyl) naphthalene were purchased from Alfa Aesar. 2-(Bromomethyl) naphthalene was purchased from Tokyo Chemical Industry Co., Ltd (TCI). Benzyl bromide was purchased from CMD Millipore. All solvents were purchased from Fisher Scientific and Acros Organics. All reagents and solvents were used as received without further purification. Melting points were obtained on a MeltTemp apparatus. Deuterated solvents were purchased from Acros Organics and used as received. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 (2.50 and 39.51 ppm, respectively) on a Varian MR-400 (^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz) spectrometer equipped with a OneNMR PZT probe. Spectra were analyzed and processed using MestReNova version 10.0.1. All NMR spectra were recorded at 25°C. Data was reported as follow: chemical shift, multiplicity (s = singlet, d = doublet, q = quartet, m = multiplet, and br = broad), and integration. MS was performed by the University of Akron Mass Spectrometry Laboratory (Akron, OH). Elemental analysis was performed by the Microanalysis Laboratory in the Department of Geology at the University of Akron (Akron, OH).

X-ray structure determination details. X-ray diffraction data were collected on a "Bruker APEX CCD" diffractometer using Mo Kα radiation (λ = 0.71073 Å). The crystal was kept at 180 K under nitrogen during unit cell and data collection. SMART (v. 5.624) was used for preliminary determination of cell constants and data collection control. Determination of integrated intensities and global cell refinement was performed with the Bruker SAINT software package, and empirical absorption correction (SADABS) was applied. The structures were solved with the ShelXS structure solution program using Direct Methods and refined with the olex2.refine refinement package using Gauss–Newton minimization [12–15]. Projections were created on Olex2.1 [14].

Cell viability protocol. ***Cell culture.*** MDA-MB-468 breast cancer cells, ATCC-HTB-132 (ATCC, Manassas, VA), and PC-3 prostate cancer cells, ATCC-CRL-1435 (ATCC), were grown according to manufacturer's instructions [16,17]. Briefly, MDA-MB-468 and PC-3 cells were grown in L-15 and DMEM/F12K media, respectively, supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic. PC-3 cells were grown in 5% CO_2 incubator at 37°C. MDA-MB-468 cells were grown in the absence of CO_2 at 37°C. Media was replaced every 3 days and split once 80% confluency was reached.

Compound solutions. All compounds were resuspended in growth media to a concentration of 1 m*M*. Solutions were then diluted 100-fold to make 100 m*M* stock solutions that were diluted for further use in the MTT assay.

MTT assays. Cell proliferation was assessed by MTT Cell Proliferation Assay, ATCC 30-1010K (ATCC), according to the manufacturer's instructions.[18] Briefly, cells were plated in triplicate at 1×10^6 cells/mL with 0.1 mL per well in 96-well plates and allowed to adhere overnight. After 24 h incubation, media was removed, and cells were treated with 0, 1, 5, 15, and 30 μ*M* of compounds **1**–**5** and 50, 40, 25, 10, and 0 μ*M* of tamoxifen solubilized in fresh complete growth media for 72 h at 37°C. After incubation, MTT reagent (ATCC) was added to each well and returned to the incubator for 4 h at 37°C to produce formazan crystals in the dark. Next, MTT detergent was added to solubilize crystals and incubated for another 2 h in the dark. Absorbance was read at 570 nm with a reference wavelength of 655 nm. IC_{50} values were calculated using the AAT Bioquest IC_{50} calculator (AAT Bioquest, Inc., Sunnyvale, CA). Standard deviations were calculated using Microsoft Excel.

Partition coefficient determination. The partition coefficient was determined using a shake flask method as described previously [19].

Synthetic procedures. ***Synthesis of 1,4-dibenzyl-1,2,4-triazolium bromide (1).*** Sodium hydroxide (0.58 g, 14.50 mmol) was added to a solution of 1,2,4-triazole (0.51 g, 7.38 mmol) in acetonitrile and methanol (9:1, v/v, 20 mL). The mixture was stirred and heated at reflux (0.5 h). Benzyl bromide (1.0 mL, 7.96 mmol) was added, and the solution was heated at reflux overnight. The reaction mixture was filtered hot *via* vacuum to remove a white precipitate, presumed to be sodium bromide. Benzyl bromide (1.0 mL, 7.96 mmol) was added to the filtrate, and the mixture was heated at reflux overnight. The solution mixture was evaporated under reduced pressure to generate a yellow colored oil, and the oil was washed with cold acetone to generate a white precipitate. The precipitate was filtered *via* vacuum, and the solids were recrystallized in hot ethanol to yield a white spindle crystalline solid **1**; yield 0.74 g (30%); mp: 168–170°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.47 (s, 1H), 9.41 (s, 1H), 7.55–7.37 (m, 10H), 5.65 (s, 2H), 5.57 (s, 2H); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ = 145.0, 142.7, 133.6, 133.2, 129.1, 128.9, 128.9, 128.9, 128.8, 54.8, 50.6. Anal. Calcd. for $C_{16}H_{16}N_3Br_1$: C, 58.19; H, 4.89; N, 12.73. Found: C, 58.19; H, 4.88; N, 12.72. HR-MS (ESI+). Calcd for $C_{16}H_{16}N_3$ [M-Br]$^+$: *m/z* 250.1339. Found *m/z* 250.1351.

Synthesis of 1,4-bis(naphthalen-2-ylmethyl)-1,2,4-triazolium bromide (2). Sodium hydroxide (0.29 g, 7.25 mmol) was added to a solution of 1,2,4-triazole (0.25 g, 3.66 mmol) in acetonitrile and methanol (9:1, v/v, 10 mL). The mixture was stirred and heated at reflux (0.5 h). 2-(Bromomethyl) naphthalene (0.84 g, 3.80 mmol) was added, and the solution was heated at reflux overnight. The reaction mixture was filtered hot to remove the white precipitate, presumed to be sodium bromide. 2-(Bromomethyl) naphthalene (0.82 g, 3.71 mmol) was added to the filtrate, and the solution was heated at reflux overnight. The resulting precipitate was collected *via* vacuum filtration and was washed with diethyl ether and air dried to provide a white powder. The product was subsequently recrystallized in ethanol to yield a white crystalline solid **2**; yield 0.98 g (62%); mp: 232–236°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.42 (s, 1H), 9.41 (s, 1H), 8.07–7.55 (m, 14H), 5.80 (s, 2H), 5.72 (s, 2H); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ = 145.1, 143.0, 132.9, 132.8, 132.7, 132.6, 130.9, 130.6, 128.9, 128.6, 128.4, 128.3, 128.0, 127.9, 127.7, 127.7, 126.9, 126.9, 126.8, 126.7, 126.2, 126.0, 55.1, 50.9. Anal. Calcd. for $C_{24}H_{20}N_3Br_1$: C, 66.98; H, 4.68; N, 9.76. Found: C, 66.15; H, 4.61; N, 9.70. HR-MS (ESI +). Calcd for $C_{24}H_{20}N_3$[M-Br]$^+$: *m/z* 350.1652. Found *m/z* 350.1666.

Crystal data for 1,4-bis(naphthalen-3-ylmethyl)-1,2,4-triazolium bromide (**2**): $C_{24}H_{20}BrN_3$, M = 430.35, monoclinic, a = 30.947(7) Å, b = 4.5974 (11) Å, c = 13.909 (3) Å, β = 94.568 (4), V = 1972.6 (8) Å^3, T = 180 K, space group *Cc*, Z = 4, 17612 reflections measured, 3486 independent reflections (R_{int} = 0.0970). The final R_1 values were 0.0546 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1077 ($I > 2\sigma(I)$). The final R_1 values were 0.0901 (all data). The final $wR(F^2)$ values were 0.1201 (all data).

Synthesis of 1,4-bis(2-(naphthalen-2-yl)-2-oxoethyl))-1,2,4-triazolium bromide (3). Potassium hydroxide (0.25 g, 4.46 mmol) was added to a solution of 1,2,4-triazole (0.25 g, 3.65 mmol) in acetonitrile and methanol (9:1, v/v, 10 mL). The mixture was stirred and heated at reflux (0.5 h). 2-(Bromoacetyl) naphthalene (0.91 g, 3.65 mmol) was added, and solution was heated at reflux overnight. The reaction mixture was filtered hot *via* vacuum to remove the precipitate presumed to be sodium bromide. 2-(Bromoacetyl) naphthalene (0.92 g, 3.70 mmol) was added to the filtrate, and the solution was heated at reflux overnight. The reaction mixture was filtered *via* vacuum filtration to generate a light brown solid. The solvent residues were removed under reduced pressure to yield a light brown powder **3**; yield 0.46 g (26%); mp: 230–233°C; FT-IR (cm^{-1}): 1685 (C=O), 1702 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.23 (s, 1H), 9.37 (s, 1H), 8.91–7.69 (m, 16H), 6.61 (s, 2H), 6.42 (s, 2H); ^{13}C{^{1}H} NMR (100 MHz, DMSO-d_6) δ = 190.3, 190.3, 145.9, 145.4, 135.6, 135.6, 132.0, 130.9, 130.8, 130.7, 130.6, 199.7, 129.5, 128.9, 128.8, 127.9, 127.5, 127.4, 123.2, 123.2, 58.5, 54.3. Anal. Calcd. for $C_{26}H_{20}N_3O_2Br_1$: C, 64.21; H, 4.15; N, 8.64. Found: C, 64.21; H, 4.14; N, 8.64. HR-MS (ESI+). Calcd for $C_{26}H_{20}N_3O_2$ [M-Br] $^+$: *m/z* 406.1550. Found *m/z* 406.1550.

Synthesis of 1,3-bis(naphthalen-3-ylmethyl)-imidazolium bromide (4). Compound **4** was synthesized according to literature procedure and used without further purification [2]. ^{1}H NMR and other characterization matched previously reported data [2].

Synthesis of 1,3-bis(naphthalen-3-ylmethyl)-benzimidazolium bromide (5). Compound **5** was synthesized according to literature procedure and used without further purification [2]. ^{1}H NMR and other characterization matched previously reported data [2].

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