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N-Phenyl-*N*′-(2-chloroethyl)ureas (CEU) as potential antineoplastic agents. Part 2: Role of ω-hydroxyl group in the covalent binding to β-tubulin

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Abstract—Tubulin is the target of many anticancer drugs, including *N*-phenyl-*N*′-(2-chloroethyl)urea (CEU). Unlike most anti-β-tubulin agents, CEUs are protein monoalkylating agents binding through their *N*′-(2-chloroethyl)urea moiety to an amino acid nearby the colchicine-binding site on β-tubulin isoform-2. Following the previously synthesized and attractive *N*-(3-ω-hydroxyalkyl-phenyl)-*N*′-(2-chloroethyl)urea that exhibited growth inhibitory activity at the nanomolar level, we investigated the importance of lower alkyl and alkoxy groups to evaluate the effect of hydroxylated group and chain length on both cell growth inhibition and the mechanism of action of CEU. Here, we describe the preparation of two new series of CEU and show that the most potent CEU derivatives beside the ω-hydroxylated **1f** were **2f** and **3e**, respectively. We have confirmed that the pentyl substituted CEUs **1f**, **2f**, and **3e** are still covalently binding to β-tubulin and still arrest cell division in G_2/M phase.

1. Introduction

Tubulin is a major target for many anticancer drug candidates, including *N*-phenyl-*N*′-(2-chloroethyl)urea (CEU).[1–3] Unlike most antimitotic agents, CEUs are protein monoalkylating agents that covalently bind through its *N*′-(2-chloroethyl)urea moiety to an amino acid neighboring the colchicine-binding site on β-tubulin isoform-2.[4,5] CEUs exhibit cell growth inhibition (GI_{50}) on numerous cancer cell lines,[4,7–16] on tumor cells having developed various chemoresistance mechanisms[16], and a potent antineoplastic activity on mice bearing colon adenocarcinoma tumors.[16] We previously investigated the effect of several structural parameters such as the presence of short electron-donating and -withdrawing groups at different position of the aromatic ring, the substitution of the aromatic ring by lower alkyl and branched-alkyl groups,[6–8] and the substitution of the *N*′-(2-chloroethyl)urea moiety on carbon 2 on the GI_{50} of CEU[13] to improve the biopharmaceutical properties of these antimitotic agents.

Recently, we published a study demonstrating that GI_{50} of CEU can be increased by a factor of 5 by substituting the position 3 of the aromatic ring of CEU by ω-hydroxylated alkyl chains, while alkynyl chain substitutes are detrimental to cell growth inhibition.[17] Here, we evaluated the effective role of the hydroxyl functional group and the side-chain length on both GI_{50} and the alkylation of β-tubulin. With the objective to answer these questions, we have developed CEUs **2a**–**f** and **3e**–**g** with the goal of assessing the role of ω-hydroxyl terminal group of the side chain substituting the aromatic ring in 3-position.

Keywords: Phenyl chloroethylurea; Antimicrotubule agents; Antitubulin agents; Antimitotic agents; Soft alkylating agents; Anticancer drugs; Colchicine-binding site ligands.
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2. Results and discussion

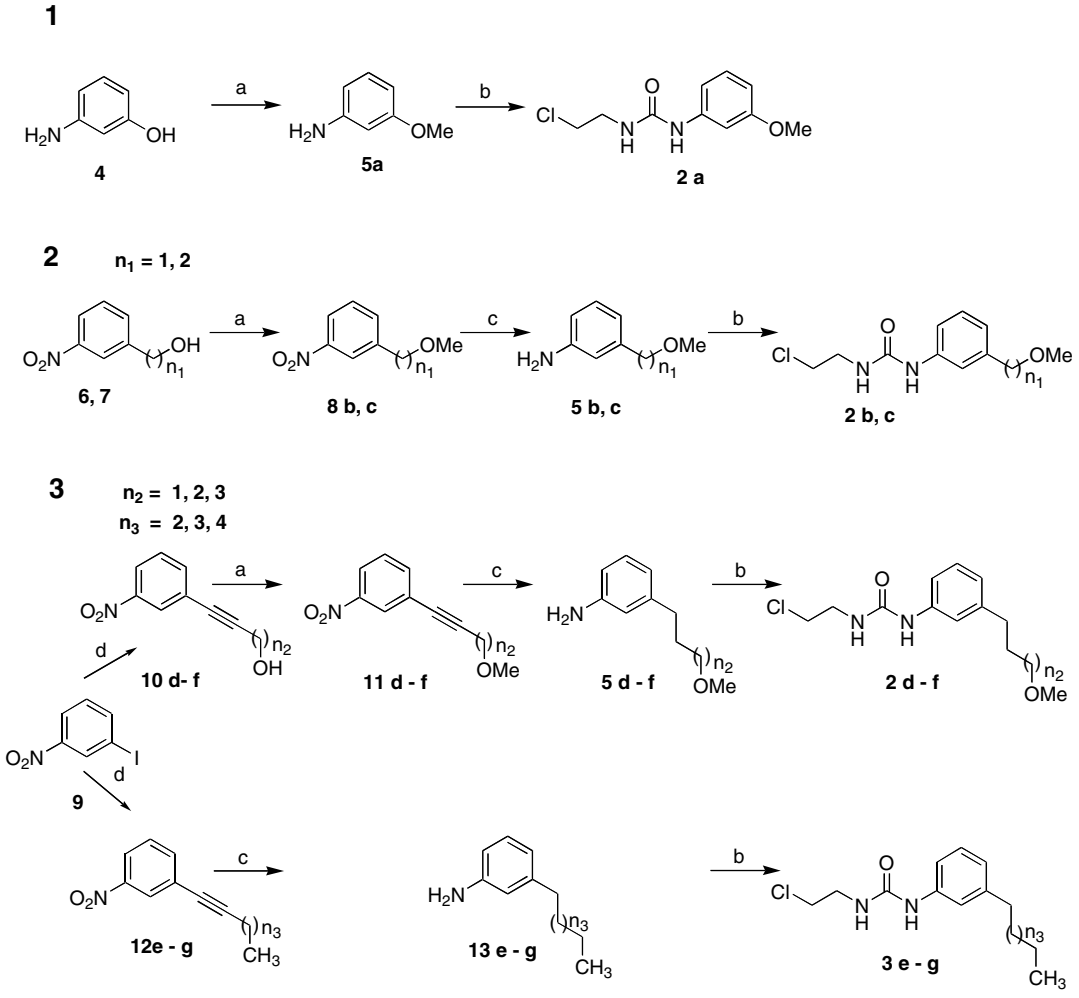
2.1. Chemistry

The homologation of the alkyl side chain at position 3 of the aromatic ring of CEU (see Scheme 1, pathway 3) was performed using a Sonogashira reaction involving the palladium-catalyzed reaction of 3-iodo nitrobenzene **9** with terminal alkynes or ω-hydroxyalkynes.[17] The reaction was co-catalyzed by Cu (I), a base, and Pd(0)/PPh_3 to produce compounds **10d–f**[17] and **12e–g**. The methylation reaction of ω-hydroxylated compounds was performed using a mixture of sodium hydride and methyl iodide to yield the methoxylated compounds **5a**, **8b**, **c**, and 11d–**f** in 45–87% yields (Scheme 1, pathways 1, 2, and 3). Catalytic hydrogenation of nitro derivatives using Pd/C produced compounds **5b-f** and **13e-g**, respectively, that were directly reacted with 2-chloroethylisocyanate to obtain the corresponding CEUs **2a–f** and **3f–h**. Compounds **1a–h**[17] and **3a**, **b**[9] have been previously described.

2.2. Tumor cell growth inhibition activity

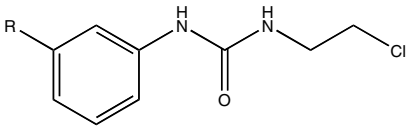
CEUs' growth inhibition activity was evaluated against three human cancer cell lines, namely human colon carcinoma HT-29, human skin melanoma M21, and human breast carcinoma MCF-7. Cell growth inhibition was assessed as described by the NCI/NIH Developmental Therapeutics Program for its drug screening program.[18] Structure–activity relationship studies were focused on the aforementioned structural parameters. The results are summarized in Table 1. GI_{50} results for **1a–h** and **3a–b** were previously described[17,9] and are shown for comparison.

We first evaluated GI_{50} effect of CEUs substituted in 3-position by lower methoxylated chains. Newly synthesized derivatives **2a–f** increased GI_{50} by 2- to 4-fold as compared to **1a–f**. Similar tendencies were also observed in the ω-alkyl series **3a**, **b**, **e–g**. However, compound **3e**, which showed an higher GI_{50} than the ω-hydroxy derivatives, may present significant in vivo advantages since it might be able to resist quick metabolic oxidative inactivation. It is of interest to mention that replacing a hydroxyl group (**1a–h**) by a methoxylated group (**2a–f**) or an alkyl group (**3a**, **b**, **e–g**) confirmed the importance of the chain length in the GI_{50} of CEU. The latter seems to be an important variable; the GI_{50} effect being optimal with a pentyl chain length substituent (see Fig. 1).



Scheme 1. Reagents: (a) NaH, MeI/THF; (b) 2-chloroethylisocyanate/DCM; (c) H_2, Pd/C/EtOH; (d) K_2CO_3, CuI, PPh_3, alcyne/1,2-DME/water.

Table 1. GI_{50} values of CEU derivatives and electrophoretic mobility shift assay of alkylated β-tubulin



Compound		Mp (°C)	Yield (%)	GI_{50} (μM)			Alkylated tubulin				
				HT-29	M21	MCF-7	2	7	17	25	48 h
1a	R = OH	117 - 119	96	10	10	10					
1b	R = CH_2-OH	106 - 109	56	10	10	10					
1c	R = $(CH_2)_2$-OH	109 - 111	20	10	10	10					
1d	R = $(CH_2)_3$-OH	96 - 98	19	5,2	6,0	7,9					
1e	R = $(CH_2)_4$-OH	72 - 74	18	1,1	1,7	2,5					
1f	R = $(CH_2)_5$-OH	92 - 95	19	0,25	0,39	0,49					
1g	R = $(CH_2)_6$-OH	83 - 84	42	0,47	0,49	0,58					
1h	R = $(CH_2)_7$-OH	86 - 88	2	0,53	0,54	0,58					
2a	R = OMe	-	81	> 10	> 10	> 10					
2b	R = CH_2-OMe	84 - 85	15	> 10	> 10	> 10					
2c	R = $(CH_2)_2$-OMe	-	1	6,4	8,6	14					
2d	R = $(CH_2)_3$-OMe	57 - 59	38	4,6	7,5	> 10					
2e	R = $(CH_2)_4$-OMe	43 - 45	1	1,7	2,3	3,9					
2f	R = $(CH_2)_5$-OMe	-	13	0,84	0,94	1,9					
3a	R = CH_3	130 - 132	38	> 10	> 10	> 10					
3b	R = CH_2-CH_3	56 - 55	60	8,9	> 10	> 10					
3e	R = $(CH_2)_4$-CH_3	-	49	0,96	0,87	1,6					
3f	R = $(CH_2)_5$-CH_3	-	48	1,1	1,4	1,0					
3g	R = $(CH_2)_6$-CH_3	-	41	1,9	1,7	2,0					

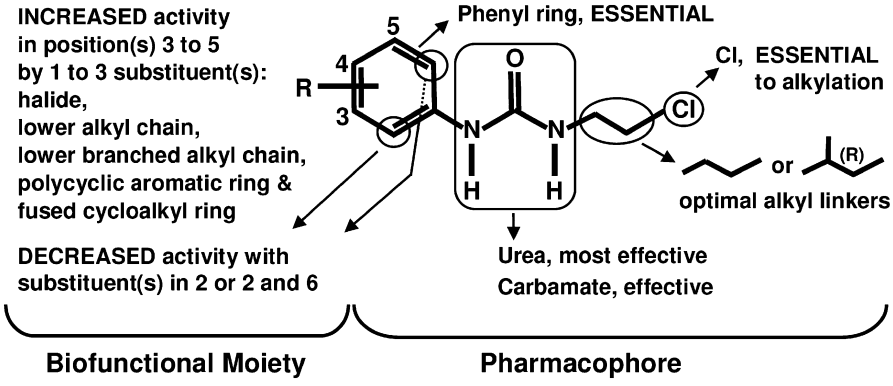


Figure 1. General formula of 1-(2-chloroethyl)-3-phenyl-urea (CEU) scaffold with key atoms and groups to insure alkylation of β-tubulin and cell growth inhibition.

2.3. Electrophoresis gel assay

The alkylating ability of compounds **1f–h**, **2e–f**, and **3e–g** on β-tubulin was examined using an electrophoretic mobility shift assay.[4,7,14,15,19] The appearance of a second β-tubulin band (lower band in the gel shown in Table 1) with an apparent faster mobility is indicative of a measurable amount of alkylated β-tubulin[4] (see Table 1). The appraisals of the covalent binding of CEU on β-tubulin were performed using MDA-MB-231 cells. Compounds **1f**, **2f**, and **3e** covalently bound β-tubulin at a concentration of 5 μM. Measurements of the relative intensity of the alkylated tubulin with respect to the non-alkylated tubulin (see Table 1) are shown in Figure 2. After 48 h, the extent of the β-tubulin-CEU by-product was maximal with **1f**, followed by its methoxylated derivative **2f** and the alkyl derivative **3e** (94%, 81%, and 90%, respectively). Measurements performed using **1f** at 30 μM indicated an alkylation rate of 50% at 6 h and 100% after 12 h.[19] The various alkylation percentage reported here may reflect CEUs' affinity for tubulin. However, these results do not provide any significant information on the kinetics of β-tubulin's alkylation by CEUs. Compounds **1f** and **2f** are the molecules displaying the highest affinity for β-tubulin. Such results have been reported previously for other CEU subsets.[4,7,14,19] The most potent CEUs are substituted by five-carbon atom substituents in 3-position of their aromatic ring. They are so far our best hit compounds with respect to β-tubulin alkylation and GI_{50}.

2.4. DNA cell cycle analysis

Antimicrotubule agents such as colchicine and vinblastine are known to block the cell cycle in G_2/M phase through microtubule disruption.[4] The analogy of action between CEUs and microtubule-disrupting agents on the cytoskeleton prompted us to examine the effects of compounds **1f**, **2f**, and **3e** on the cell cycle. Exponentially growing M21 cells were treated with compounds **1f**, **2f**, and **3e** or DMSO for escalating period of time as described in Section 4. Flow cytometric analysis showed that compounds **1f**, **2f**, and **3e** caused a significant accumulation of cells in G_2/M phase. It suggests that **1f**, **2f**, and **3e** may induce microtubule disruption and consequently prevent mitosis in a similar fashion to colchicine and vinblastine (see Table 2).

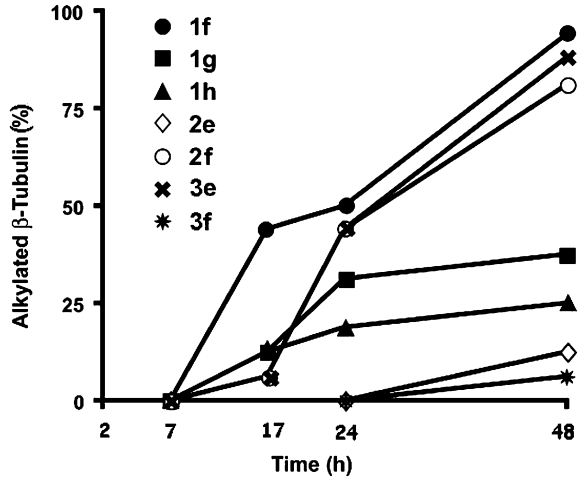


Figure 2. Extent of β-tubulin alkylation at escalating time of exposure with the drug as determined from optical density measurement of electrophoretic mobility shift assay measurements presented in Table 1.

Table 2. Quantification of the FACS analyses for compounds **1f**, **2f**, and **3e**

Compound	Concn (μM)	Apoptotic cells and cell cycle phase (% of population)			
		Apoptosis	G0/G1	S	G_2/M
1f	1.2	7.58	54.35	16.65	21.82
	4	9.83	7.57	12.12	70.24
	10	10.71	5.9	12.11	10.71
2f	3	8.5	31.28	19.98	40.22
	10	9.02	7.52	14.37	68.57
	25	8.63	6.74	13.79	70.84
3e	3	6.16	15.06	17.75	60.78
	10	8.59	7.85	13.28	68.06
	25	7.84	8.75	15.23	68.48
DMSO		3.04	61.36	20.05	15.88

3. Conclusion

We report here the results of our investigation on the role of terminal ω-hydroxyl functional group on biological activity of a series of CEUs (compounds **1a–h**[17]). We have also prepared two new series of CEUs by modifying the functional group on side chain. Structure–activity relationships show that active CEUs, substituted in 3-position, require for low concentration cell growth inhibition a lower alkyl substituted or not by either an hydroxyl or a lower alkoxyl group; the cell growth inhibition being optimal with a five-carbon atom chain. The best compounds, namely **1f**, **2f**, and **3e**, were still potent microtubule disruptors by covalently binding to β-tubulin isoform-2. We therefore conclude that other appropriate terminal groups might optimize the biological activity of CEUs. Indeed, it is known that the binding of colchicinoid derivatives such as MTC, TCB, and TKB to tubulin relies on the contribution of presence of carbonyl group on ring C (or C′) to generate a specific role in the inhibition process.[20,21] The results of further optimization of this series with contribution of carbonyl terminal group on the side chain will be reported in due course.

4. Experimental Section

4.1. Biological assays and reagents

Biochemicals, drugs, and the monoclonal antibody anti-β-tubulin (clone TUB 2.1) were purchased from Sigma Chemical (St. Louis, MO). The bovine calf serum was obtained from Hyclone (Road Logan, UT). The ECL

Western blotting detection reagent kit was provided by Amersham Canada (Oakville, Canada). Mixtures are expressed as volume/volume ratios unless otherwise indicated. All drugs were dissolved in DMSO, and the final concentration of DMSO in the culture medium was maintained at 0.5%.

4.1.1. Cell culture and growth inhibition activity. The growth inhibition potency of CEUs was assessed using the procedure described by the National Cancer Institute for its drug screening program.[18] 96-Well tissue culture plates were seeded with 100 μL of tumor cell lines suspended in high glucose DMEM supplemented with 5% (v/v) defined bovine calf serum iron supplemented (Hyclone). Plates were incubated at 37 °C, 5% CO_2 for 24 h. Freshly solubilized drugs in DMSO were diluted in fresh medium and aliquots of 100 μL containing sequential dilution of drugs were added. Final drug concentrations ranged from 10 to 0.3 μM. DMSO concentration was maintained lower than 0.5% to avoid solvent's cytotoxicity. Plates were incubated for 48 h. Assays were stopped by addition of cold trichloroacetic acid to the wells (final concentration was 10%), followed by incubation for 60 min at 4 °C. Plates were washed five times with tap water. Sulforhodamine B solution (50 μL) at 0.1% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 15 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid. Bound stain was solubilized with 10 mM Tris base, and the absorbance was read using a μQuant Universal Microplate Spectrophotometer (Biotek, Winooski, VT) at 585 nm. The results were compared with those of a control reference plate fixed on the treatment day and the growth inhibition percentage was calculated for each drug contact period. The experiments were performed at least twice in triplicate. The GI_{50} assay was considered valid when the variability among data for a given set of conditions, within the same experiment, was less than 10% with respect to the mean value.

4.1.2. Electrophoretic mobility shift assay to evaluate β-tubulin alkylation. Exponentially growing MDA-MB-231 cells (2.2×10^5) were plated in 12-well plates and incubated overnight at 37 °C. The cells were then treated with a 5 μM test compound solution, except for the control, for 2, 7, 17, 25, and 48 h, and were harvested directly in culture medium using a rubber policeman. Cells were centrifuged, and the pellets were washed with 500 μL of cold PBS. After centrifugation, the pellets were lysed using Laemmli sample buffer. Samples (5×10^4 cells) were analyzed by 10% SDS–PAGE using the Laemmli system. Membranes were then incubated with PBSMT (PBS, pH 7.4, 5% fat-free dried milk and 0.1% Tween 20™) for 1 h at room temperature and then with 1/500 monoclonal anti-β-tubulin (clone TUB 2.1) for 1 h. Membranes were washed with PBSMT and incubated with 1/2500 peroxidase-conjugated antimouse immunoglobulin in PBSMT for 30 min. Detection of the immunoblot was carried out with the ECL Western blotting detection reagent kit. All data were obtained from the same experiment. Duplicate experiments were conducted on some compounds and showed similar results. Optical densities of the normal and alkylated β-tubulin band were integrated using a NIH imager (Scion Corporation, Frederick, MD) and the percentage of alkylated β-tubulin was calculated.

4.1.3. Cell cycle analysis. After incubation of HT 1080 cells with drug, the cells were harvested, resuspended in 1 mL PBS, and fixed by the addition of 2.4 mL of ice-cold anhydrous ethanol. Then, 5×10^5 cells from each sample were centrifuged for 3 min at 1000*g*. Cell pellets were resuspended in PBS containing 50 μg/mL of propidium iodide and 40 U/mL of RNase A (Boehringer Mannheim, Laval, Canada). Mixtures were incubated at room temperature for 30 min, and cell cycle distribution was analyzed using an Epics Elite ESp flow cytometer (Coulter Corporation, Miami, FL). Quantification of the FACS analyses was carried out with the software developed by Scripps Research Institute (http://www.scripps.edu/e_index.html).

4.2. Experimental procedures

4.2.1. Chemistry and chemical methods. Proton NMR spectra were recorded on a Brucker AM-300 spectrometer (Bruker, Germany). Chemical shifts (δ) are reported in parts per million relative to the internal tetramethylsilane standard. IR spectra were recorded on a Unicam spectrometer. Uncorrected melting points were determined on an Electrothermal melting point apparatus. ESIMS spectral analysis was carried out at the Mass Spectroscopy Laboratory of Molecular Medicine Research Centre, Medical Sciences Bldg, University of Toronto (http://www.medresearch.utoronto.ca/pmsc_home.html). All reactions were conducted under a rigorously dried nitrogen atmosphere. Chemicals were supplied by Aldrich Chemical Co. (Milwaukee, WI). The aromatic nitro alkynols **10d–f** and ω-hydroxylated alkyl CEUs **1a–h** were prepared as previously described.[17] Liquid flash chromatography was performed on silica gel 60 A (American Chemicals Ltd., Montreal, Canada), using the indicated solvent mixture expressed as volume/volume ratios. Solvents and reagents were used without purification unless specified otherwise. The progress of all reactions was monitored using TLC on precoated silica gel plates (Merck Silica Gel 60 F_{254}). The chromatograms were visualized under UV light at 254 nm. For column chromatography Merck Silica Gel (70–230 mesh) was used.

4.3. General preparation of compounds 1a–h[17] and 3a, b[9]

Syntheses and characterizations of compounds **1a–h**[17] were previously reported. Briefly, ω-hydroxylated CEUs **1a–h** were synthetized by nucleophilic addition of the ω-hydroxyl alkylphenylamines on 2-chloroethylisocyanate. The ω-hydroxylated alkenylphenylamines **10d–h**[17] were reduced under hydrogen atmosphere to afford the hydroxylated alkylphenylamines. Compounds **10d–h** were synthesized by a Sonogashira coupling between 1-iodo-3-nitrobenzene and ω-hydroxylated alkynyls. CEUs **3a**, **b** were directly prepared by the nucleophilic addition of 1-methyl or 1-ethyl-3-aniline to 2-chloroethylisocyanate.

4.3.1. *N*-[3-(4-Hydroxybutyl)phenyl]-*N*′-(2-chloroethyl) urea (1e). The preparation of compound **1e** was previously reported.[17] ESIMS (*m*/*z*) 295.2 $[M+2+Na]^+$, 293.2 $[M+Na]^+$, 273.2 $[M+2]^+$, 271.2 $[M]^+$.

4.3.2. *N*-[3-(5-Hydroxypentyl)phenyl]-*N*′-(2-chloroethyl) urea (1f). The preparation of compound **1f** was previously reported.[17] ESIMS (*m*/*z*) 309.2 $[M+2+Na]^+$, 307.2 $[M+Na]^+$, 287.2 $[M+2]^+$, 285.2 $[M]^+$.

4.3.3. *N*-[3-(6-Hydroxyhexyl)phenyl]-*N*′-(2-chloroethyl) urea (1g). The preparation of compound **1g** was previously reported.[17] ESIMS (*m*/*z*) 323.2 $[M+2+Na]^+$, 321.2 $[M+Na]^+$, 301.2 $[M+2]^+$, 299.2 $[M]^+$.

4.3.4. *N*-[3-(7-Hydroxyheptyl)phenyl]-*N*′-(2-chloroethyl) urea (1h). The preparation of compound **1h** was previously reported.[17] ESIMS (*m*/*z*) 337.2 $[M+2+Na]^+$, 335.2 $[M+Na]^+$, 315.2 $[M+2]^+$, 313.2 $[M]^+$.

4.4. General preparation of compounds 2a–f and 3e–g

4.4.1. 1-(2-Chloroethyl)-3-(3-methoxyphenyl)urea (2a). 2-Chloroethylisocyanate (1.640 mmol) was added dropwise to a cold solution (ice bath) of **5a** (1.370 mmol) in dry dichloromethane (15 mL/g of aniline). The ice bath was then removed and the reaction mixture was stirred at room temperature for 20 h. After completion of the reaction, the solvent was evaporated under reduced pressure to give an off-white solid, which was purified by flash chromatography (silica gel, ethyl acetate:hexanes (6/4)). Yield: 91%; IR (KBr) ν 3307, 1632, 1248 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.12 (br s, 1H, NH), 7.21 (m, 3H, Ar), 6.87 (d, 1H, $J = 7.0$, Ar), 3.97 (s, 3H, CH_3), 3.57 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.1, 139.4, 138.0, 129.2, 125.1, 122.1, 118.4, 57.8, 44.5, 42.1.

4.4.2. 1-(2-Chloroethyl)-3-(3-(1-methoxymethyl)phenyl) urea (2b). Compound **2b** was synthesized from **5b**. The crude product was purified by flash chromatography (silica gel, ethyl acetate:hexanes (6/4)). Yield: 87%; mp 84–85 °C; IR (KBr) ν 3330, 1636, 1245 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.29 (m, 3H, Ar), 7.04 (m, 1H, Ar), 4.40 (s, 2H, CH_2), 3.57 (m, 4H, CH_2), 3.38 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 155.8, 139.4, 138.6, 129.3, 123.1, 120.1, 120.0, 74.4, 58.2, 44.8, 42.2.

4.4.3. 1-(2-Chloroethyl)-3-(3-(2-methoxyethyl)phenyl) urea (2c). Compound **2c** was synthesized from **5c**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 81%; IR (NaCl) ν 3329, 1640, 1246 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.87 (br s, 1H, NH), 7.21 (m, 3H, Ar), 6.97 (d, 1H, $J = 7.0$, Ar), 3.58 (m, 2H, CH_2), 3.51 (m, 5H, CH_2, CH_3), 2.61 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.4, 143.2, 138.7, 129.2, 123.4, 120.2, 118.0, 71.9, 44.5, 44.4, 32.2, 29.9.

4.4.4. 1-(2-Chloroethyl)-3-(3-(3-methoxypropyl)phenyl) urea (2d). Compound **2d** was synthesized from **5d**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 81%; mp 57–59 °C; IR (KBr) ν 3320, 1627, 1237 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.67 (br s, 1H, NH), 7.15 (m, 3H, Ar), 6.85 (d, 1H, $J = 7.0$, Ar), 3.51 (m, 4H, CH_2), 3.34 (m, 2H, CH_2), 2.58 (m, 2H, CH_2), 1.82 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.4, 143.2, 138.6, 129.1, 123.7, 120.5, 118.0, 71.9, 58.5, 44.5, 42.10, 32.3, 31.0.

4.4.5. 1-(2-Chloroethyl)-3-(3-(4-methoxybutyl)phenyl) urea (2e). Compound **2e** was synthesized from **5e**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 77%; mp 43–45 °C; IR (KBr) ν 3328, 1639, 1250 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.57 (br s, 1H, NH), 7.14 (m, 3H, Ar), 6.84 (d, 1H, $J = 7.0$, Ar), 3.64 (m, 4H, CH_2), 3.47 (m, 2H, CH_2), 3.35 (s, 3H, CH_3), 2.54 (m, 2H, CH_2), 1.60 (m, 4H, CH_2), 1.24 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.3, 143.7, 138.6, 129.0, 123.7, 120.5, 117.9, 72.7, 58.5, 44.6, 42.1, 35.7, 29.2, 27.8. ESIMS (*m*/*z*) 309.2 $[M+2+Na]^+$, 307.2 $[M+Na]^+$, 287.3 $[M+2]^+$, 285.2 $[M]^+$.

4.4.6. 1-(2-Chloroethyl)-3-(3-(5-methoxypentyl)phenyl) urea (2f). Compound **2f** was synthesized from **5f**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 27%; IR (NaCl): ν 3333, 1640, 1246 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.18 (br s, 1H, NH), 7.14 (m, 3H, Ar), 6.86 (d,, 1H, $J = 7.0$, Ar), 3.57 (m, 4H, CH_2), 3.33 (m, 4H, CH_2), 2.55 (t, 2H, $J = 7.0$, CH_2), 1.59 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$) δ 156.0, 144.0, 138.4, 129.1, 123.9, 120.8, 118.2, 58.5, 44.6, 42.0, 35.9, 31.2, 29.5, 25.9. ESIMS (*m*/*z*) 323.2 $[M+2+Na]^+$, 321.2 $[M+Na]^+$, 301.3 $[M+2]^+$, 299.3 $[M]^+$.

4.4.7. 1-(2-Chloroethyl)-3-(3-pentylphenyl)urea (3e). Compound **3e** was synthesized from **13e**. The crude product was purified by flash chromatography (silica gel, hexanes:ethyl acetate (6/4)). Yield: 89%; IR (KBr) ν 3340, 2932, 1685, 1247 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.94 (br s, 1H, NH), 7.09 (m, 4H, Ar), 6.77 (br s, 1H, NH), 2.48 (t, 2H, $J = 7.5$, CH_2), 1.53 (m, 2H, CH_2), 1.22 (m, 4H, CH_2), 0.81 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 156.4, 143.9, 138.9, 128.7, 123.1, 119.9, 117.2, 36.9, 31.5, 31.0, 22.5, 14.5, 14.0. ESIMS (*m*/*z*) 294.2 $[M+2+Na]^+$, 292.2 $[M+Na]^+$, 271.2 $[M+2]^+$, 269.3 $[M]^+$.

4.4.8. 1-(2-Chloroethyl)-3-(4-hexylphenyl)urea (3f). Compound **3f** was synthesized from **13f**. The crude product was purified by flash chromatography (silica gel, hexanes:ethyl acetate (6/4)). Yield: 86%; IR (KBr) ν 3326, 2938, 1698, 1251 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.11 (br s, 1H, NH), 7.07 (m, 4H, Ar), 6.76 (br s, 1H, NH), 2.49 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 1.25 (m, 6H, CH_2), 0.78 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 156.0, 144.0, 138.9, 128.7, 123.1, 119.8, 117.1, 35.9, 31.6, 31.3, 29.0, 22.5, 14.0. ESIMS (*m*/*z*) 307.3 $[M+2+Na]^+$, 305.3 $[M+Na]^+$, 285.2 $[M+2]^+$, 283.2 $[M]^+$.

4.4.9. 1-(2-Chloroethyl)-3-(4-heptylphenyl)urea (3g). Compound **3g** was synthesized from **13g**. The crude product was purified by flash chromatography (silica gel, hexanes:ethyl acetate (6/4)). Yield: 91%; IR (KBr) ν 3326, 2927, 1698, 1250 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13 (br s, 1H, NH), 7.11 (m, 4H, Ar), 6.79 (br s, 1H, NH), 2.48 (t, 2H, $J = 7.5$, CH_2), 1.53 (m, 2H, CH_2),

1.20 (m, 8H, CH_2), 0.82 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 156.0, 144.1, 138.8, 128.8, 123.3, 120.0, 117.2, 36.0, 31.8, 31.4, 29.3, 29.1, 22.6, 14.1. ESIMS (*m/z*) 322.3 $[M+2+Na]^+$, 319.2 $[M+Na]^+$, 299.3 $[M+2]^+$, 297.4 $[M]^+$.

4.5. General preparation of compounds 5b–f and 13e–g

A mixture of the appropriate alkenyl (0.43 mmol), Pd/C 10% dissolved in ethanol (30 mL) was reduced under hydrogen atmosphere (38 psi) overnight. The catalyst was removed by filtration on Celite and the filtrate was evaporated to dryness. The residue was purified by flash chromatography on silica gel to afford **5b–f** or **13e–g**.

4.5.1. 3-(1-Methoxymethyl)phenylamine (5b). Compound **5b** was synthesized from **8b**. The crude product was purified by flash chromatography (silica gel, ethyl acetate:EtOH (9/1)). Yield: 23%; IR (NaCl) ν 3323, 2922, 1097 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.25 (s, 1H, Ar), 7.12 (t, 1H, $J = 7$, Ar), 6.65 (m, 2H, Ar), 4.36 (s, 2H, CH_2), 3.65 (br s, 2H, NH), 3.36 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 146.2, 139.6, 129.4, 118.1, 114.7, 114.4, 72.0, 58.1.

4.5.2. 3-(2-Methoxyethyl)phenylamine (5c). Compound **5c** was synthesized from **8c**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 22%; IR (NaCl) ν 3359, 2925, 1112 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.26 (s, 1H, Ar), 7.15 (m, 2H, Ar), 6.91 (d, 1H, $J = 7$, Ar), 3.47 (m, 5H, CH_2, CH_3), 2.41 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 146.1, 143.8, 129.2, 119.1, 115.3, 112.2, 72.0, 58.5, 32.8.

4.5.3. 3-(3-Methoxypropyl)phenylamine (5d). Compound **5d** was synthesized from **11d**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 78%; IR (NaCl) ν 3310, 2928, 1111 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.07 (t, 1H, $J = 7.5$, Ar), 6.60 (d, 1H, $J = 8$, Ar), 6.53 (m, 2H, Ar), 3.68 (br s, 2H, NH), 3.36 (m, 5H, NH, CH_2), 2.59 (t, 2H, $J = 7$, CH_2), 1.86 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 146.1, 143.3, 129.3, 119.0, 115.5, 112.9, 72.0, 58.5, 32.3, 31.1.

4.5.4. 3-(4-Methoxybutyl)phenylamine (5e). Compound **5e** was synthesized from **11e**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 35%; IR (NaCl) ν 3390, 2916, 1116 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.04 (t, 1H, $J = 7.5$, Ar), 6.59 (d, 1H, $J = 8$, Ar), 6.51 (m, 2H, Ar), 3.62 (br s, 2H, NH), 3.37 (m, 2H, CH_2), 3.31 (s, 3H, CH_3), 2.53 (t, 2H, $J = 7$, CH_2), 1.42 (m, 4H, CH_2); ^{13}C NMR ($CDCl_3$) δ 146.0, 143.7, 129.2, 119.1, 115.5, 112.8, 72.7, 58.5, 35.8, 29.7, 27.8.

4.5.5. 3-(1-Methoxypentyl)phenylamine (5f). Compound **5f** was synthesized from **11f**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 85%; IR (NaCl) ν 3366, 2928, 1110 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.07 (t, 1H, $J = 7.5$, Ar), 6.49 (d, 1H, $J = 8$, Ar), 6.43 (m, 2H, Ar), 3.31 (m, 5H, CH_2, CH_3), 2.51 (m, 2H, CH_2), 1.52 (m, 4H, CH_2), 1.32 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 146.3, 143.9, 129.1, 118.9, 114.3, 11.5, 72.8, 58.5, 36.0, 31.3, 29.5, 22.8.

4.5.6. 3-Pentylphenylamine (13e). Compound **13e** was synthesized from **12e**. The crude product was purified by flash chromatography (silica gel, CH_2Cl_2:EtOH (95/5)). Yield: 93%; IR (NaCl) ν 3335, 297, 1670, 1489 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.09 (t, 1H, $J = 7.9$, Ar), 6.63 (d, 1H, $J = 7.5$, Ar), 6.53 (m, 2H, Ar), 3.56 (br s, 2H, NH_2), 2.54 (m, 2H, CH_2), 1.63 (pent, 2H, $J = 7.0$, CH_2), 1.36 (m, 4H, CH_2), 0.93 (t, 3H, $J = 7.0$, CH_3); ^{13}C NMR ($CDCl_3$) δ 146.3, 144.3, 129.1, 118.9, 115.4, 112.6, 36.0, 31.6, 31.1, 22.6, 14.1.

4.5.7. 3-Hexylphenylamine (13f). Compound **13f** was synthesized from **12f**. The crude product was purified by flash chromatography (silica gel, CH_2Cl_2:EtOH (95/5)). Yield: 97%; IR (NaCl) ν 3366, 2927, 1618, 1460 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.11 (t, 1H, $J = 7.5$, Ar), 6.64 (d, 1H, $J = 7.4$, Ar), 6.54 (m, 2H, Ar), 3.57 (br s, 2H, NH_2), 2.57 (m, 2H, CH_2), 1.64 (m, 2H, CH_2), 1.36 (m, 6H, CH_2), 0.95 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 146.3, 144.3, 129.2, 118.9, 115.4, 112.6, 36.1, 31.4, 29.1, 22.7, 14.2.

4.5.8. 3-Heptylphenylamine (13g). Compound **13g** was synthesized from **12g**. The crude product was purified by flash chromatography (silica gel, CH_2Cl_2:EtOH (95/5)). Yield: 82%; IR (NaCl) ν 3374, 2926, 1617, 1459 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.08 (t, 1H, $J = 8.0$, Ar), 6.61 (d, 1H, $J = 7.5$, Ar), 6.53 (m, 2H, Ar), 3.50 (br s, 2H, NH_2), 2.53 (m, 2H, CH_2), 1.62 (m, 2H, CH_2), 1.29 (m, 8H, CH_2), 0.90 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 146.0, 144.3, 129.1, 119.1, 115.5, 112.7, 36.0, 31.9, 31.4, 29.4, 29.2, 22.7, 14.1.

4.6. General preparation of compounds 5a, 8b–f, and 11d–f

4.6.1. 3-Methoxybenzenamine (5a). NaH (60%) (97.40 mg, 4.06 mmol) was suspended in dry THF (8 mL). A solution of compound **4** (0.98 mmol) in dry THF (3 mL) was added dropwise at 0 °C. The mixture was stirred for 15 min at 0 °C. MeI (2.34 mmol) was added dropwise and the mixture was stirred at room temperature for 3 h. Saturated solution of $NaHCO_3$ (10 mL) and MeOH (10 mL) were then added. The mixture was extracted with AcOEt (3× 15 mL), dried over Na_2SO_4, filtered, evaporated to dryness, and purified by flash chromatography (silica gel, ethyl acetate). Yield: 87%; IR (NaCl) ν 1532, 1114 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.18 (s, 1H, Ar), 8.12 (d, 1H, $J = 8.0$, Ar), 7.63 (d, 1H, $J = 7.5$, Ar), 7.51 (t, 1H, $J = 8.0$, Ar), 3.38 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 148.4, 140.6, 133.3, 129.3, 122.5, 122.2, 58.5.

4.6.2. 1-(1-Methoxymethyl)-3-nitrobenzene (8b). Compound **8b** was synthesized from **6**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 75%; IR (NaCl) ν 1535, 1115 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.16 (s, 1H, Ar), 8.09 (d, 1H, $J = 8.0$, Ar), 7.63 (d, 1H, $J = 7.5$, Ar), 7.48 (t, 1H, $J = 8.0$, Ar), 4.51 (s, 2H, CH_2), 3.40 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 148.4, 140.6, 133.3, 129.3, 122.5, 122.2, 73.3, 58.5.

4.6.3. 1-(2-Methoxyethyl)-3-nitrobenzene (8c). Compound **8c** was synthesized from **7**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 81%; mp >310 °C; IR (KBr) ν 1592, 1162 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.13 (s, 1H, Ar), 8.05 (d, 1H, $J = 8.0$, Ar), 7.72 (d, 1H, $J = 8.0$, Ar), 7.60 (m, 1H, Ar), 3.62 (t, 2H, $J = 7.0$, CH_2), 3.28 (s, 3H, CH_3), 2.92 (t, 2H, $J = 7.0$, CH_2); ^{13}C NMR (DMSO-d_6) δ 148.7, 138.1, 129.2, 126.9, 124.3, 123.9, 73.3, 58.5, 14.8.

4.6.4. 1-(3-Methoxyprop-1-ynyl)-3-nitrobenzene (11d). Compound **11d** was synthesized from **10d**. The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 61%; IR (NaCl) ν 2235, 1346 (NO_2), 1091 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.27 (s, 1H, Ar), 8.15 (d, 1H, $J = 8.0$, Ar), 7.73 (d, $J = 8.0$, Ar), 7.49 (t, 1H, $J = 8.0$, Ar), 7.73 (d, 1H, $J = 8.0$, Ar), 7.49 (t, 1H, $J = 8.0$, Ar), 3.59 (s, 2H, CH_2), 3.37 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 148.3, 137.5, 129.5, 127.4, 124.2, 123.3, 87.9, 83.9, 60.2, 57.9, 29.7.

4.6.5. 1-(4-Methoxybut-1-ynyl)-3-nitrobenzene (11e). Compound **11e** was synthesized from **10e**.[17] The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 67%; IR (NaCl) ν 2223, 1353, 1109 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.23 (s, 1H, Ar), 8.10 (d, 1H, $J = 8.0$, Ar), 7.68 (d, 1H, $J = 7.0$, Ar), 7.44 (t, 1H, $J = 8.0$, Ar), 3.59 (t, 2H, $J = 7.0$, CH_2), 3.40 (s, 3H, CH_3), 2.70 (t, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 148.1, 137.4, 129.1, 126.5, 125.6, 122.5, 90.0, 79.3, 70.5, 58.8, 19.7.

4.6.6. 1-(5-Methoxypent-1-ynyl)-3-nitrobenzene (11f). Compound **11f** was synthesized from **10f**.[17] The crude product was purified by flash chromatography (silica gel, ethyl acetate). Yield: 58%; IR (NaCl) ν 2228, 1353, 1116 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.18 (s, 1H, Ar), 8.07 (d, 1H, $J = 8.0$, Ar), 7.64 (d, 1H, $J = 8.0$, Ar), 7.42 (t, 1H, $J = 8.0$, Ar), 3.49 (t, 2H, $J = 7.0$, CH_2), 3.34 (s, 3H, CH_3), 2.49 (t, 2H, $J = 7.0$, CH_2), 1.86 (m, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 148.1, 137.3, 129.1, 126.3, 125.8, 122.3, 97.7, 78.7, 71.1, 58.6, 29.7, 16.1.

4.7. General preparation of compounds 12e–g

4.7.1. 1-Nitro-3-pentynylbenzene (12e). To a mixture of the compound **9** (4.56 mmol), K_2CO_3 (1.57 g, 11.4 mmol) in a mixture of 1,2-DME/water (1:1; 30 mL) were successively added CuI (34 mg, 0.18 mmol), PPh_3 (95.80 mg, 0.36 mmol), and Pd/C 10% (97.5 mg, 0.09 mmol). The mixture was stirred at room temperature for 1 h. Afterward, 4-pentynoic acid (14.40 mmol) was added, and the mixture was refluxed overnight. After cooling, the mixture was filtered on Celite and the solvent was evaporated under reduced pressure. An aqueous solution of 1 N HCl (20 mL) was then added to the residue. The aqueous solution was extracted with ethyl acetate (3× 15 mL). The organic extracts were combined, washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure. The crude product was purified by flash chromatography (silica gel, CH_2Cl_2:EtOH (95/5)). Yield: 59%; IR (NaCl) ν 3084, 2934, 2225, 1530, 1350 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.16 (s, 1H, Ar), 8.06 (d, 1H, $J = 8.3$, Ar), 7.63 (d, 1H, $J = 7.5$, Ar), 7.41 (t, 1H, $J = 8.0$, Ar), 2.37 (t, 2H, $J = 7.0$, CH_2), 1.62 (m, 2H, CH_2), 1.01 (m, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 148.1, 137.3, 129.1, 126.3, 125.9, 122.2, 93.4, 78.6, 22.1, 21.8, 21.3.

4.7.2. 1-Nitro-3-hexynylbenzene (12f). Compound **12f** was synthesized from **9** and 1-hexyne. The crude product was purified by flash chromatography (silica gel, hexanes:ethyl acetate (60/40)). Yield: 58%; IR (NaCl) ν 3083, 2933, 2229, 1531, 1350 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.21 (s, 1H, Ar), 8.09 (d, 1H, $J = 8.0$, Ar), 7.67 (d, 1H, $J = 7.8$, Ar), 7.44 (t, 1H, $J = 7.9$, Ar), 2.42 (t, 2H, $J = 7.0$, CH_2), 1.61 (m, 2H, CH_2), 1.47 (m, 2H, CH_2), 0.93 (t,, 3H, $J = 7.0$, CH_3); ^{13}C NMR ($CDCl_3$) δ 148.1, 137.4, 129.2, 126.3, 125.8, 122.3, 93.4, 78.6, 22.1, 21.8, 21.5, 21.3.

4.7.3. 1-Nitro-3-heptynylbenzene (12g). Compound **12g** was synthesized from **9** and 1-heptyne. The crude product was purified by flash chromatography (silica gel, hexanes:ethyl acetate (60/40)). Yield: 55%; IR (NaCl) ν 3447, 2932, 2227, 1532, 1351 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.17 (s, 1H, Ar), 8.06 (d, 1H, $J = 7.9$, Ar), 7.63 (d, 1H, $J = 7.6$, Ar), 7.41 (t, 1H, $J = 8.0$, Ar), 2.39 (t, 2H, $J = 7.0$, CH_2), 1.55 (m, 2H, CH_2), 1.41 (m, 4H, CH_2), 0.91 (t, 3H, $J = 7.0$, CH_3); ^{13}C NMR ($CDCl_3$) δ 148.1, 137.3, 129.1, 126.3, 126.0, 122.1, 93.6, 78.5, 31.1, 28.2, 22.2, 19.3, 13.9.

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