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Article

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Selective Inhibition of *E. coli* RNA and DNA Topoisomerase I by Hoechst 33258 Derived Mono and Bisbenzimidazoles

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ABSTRACT:

A series of Hoechst 33258 based mono and bisbenzimidazoles have been synthesized and their *E. coli* DNA topoisomerase I inhibition, binding to B-DNA duplex and antibacterial activity has been evaluated. Bisbenzimidazoles with alkynyl side chains display excellent *E. coli* DNA topoisomerase I inhibition properties with IC_{50} values < 5.0 μM. Several bisbenzimidazoles (**3, 6, 7, 8**) also inhibit RNA topoisomerase activity of *E. coli* DNA topoisomerase I. Bisbenzimidazoles inhibit antibacterial growth much better than mono-benzimidazoles for gram positive strains. The minimum inhibitory concentration (MIC) was much lower for Gram positive bacteria (*Enterococcus spp*. and *Staphylococcus spp* including two MRSA strains 0.3-8 μg/mL) than for the majority of Gram negative bacteria (*P.aeruginosa*, 16-32 μg/mL, *K. pneumoniae* >32μg/mL). Bisbenzimidazoles showed varied stabilization of B-DNA duplex (8.0-22.9^{o}C), and cytotoxicity studies show similar variation, dependent upon the side chain length. Modeling studies suggest critical interactions between the side chain and active site amino acids.

KEYWORDS: Topoisomerase, Hoechst 33258, bisbenzimidazole, antibacterial, DNA binding

INTRODUCTION

DNA topoisomerases are important class of enzymes that help in regulating DNA topology.[1] They are involved in several cellular functions such as removing supercoils, strand breakage during recombination, chromosome condensation as well as disentangling of intertwined DNA.[2-5] Eukaryotic DNA topoisomerases I and II have gained significant attention as drug targets particularly in cancer treatment.[6-8] On the other hand, bacterial DNA gyrase and topoisomerase IV have been targets of some established antibiotics.[9-11] Therefore, controlling DNA topoisomerase functions has been envisioned for developing new anticancer and antibacterial agents.[12,13] A number of small molecules have been tested for their ability as poisons of DNA topoisomerase functions.[14] The therapeutic interest in the development of small molecules as inhibitors of DNA topoisomerase lies in their ability to act as DNA cleavage complex stabilizing agents[12] and to recognize ATP binding site.[15] The emergence of resistance to anti-bacterials has necessitated the search of novel molecules that could help tackle these issues. Small molecules that are both DNA binders and non-binders have been known to poison the functions of DNA topoisomerases.[14] A classic DNA topoisomerase poison camptothecin is believed to act by forming a ternary complex with the topoisomerase and DNA.[16] Several other planar small molecules (both DNA intercalators and non-intercalators) have been shown to inhibit the functions of DNA topoisomerases.[17-24] Both symmetric and asymmetric bisbenzimidazoles have recently been reported as effective antibacterial agents and DNA topoisomerase poisons.[25, 26] Although bisbenzimidazoles derived from Hoechst 33258 have long been known for their B-DNA binding[27, 28] and topoisomerase I poisoning,[29-33] recent reports by us and others have identified some of them for their selectivity towards bacterial DNA topoisomerase I inhibition.[34, 35]

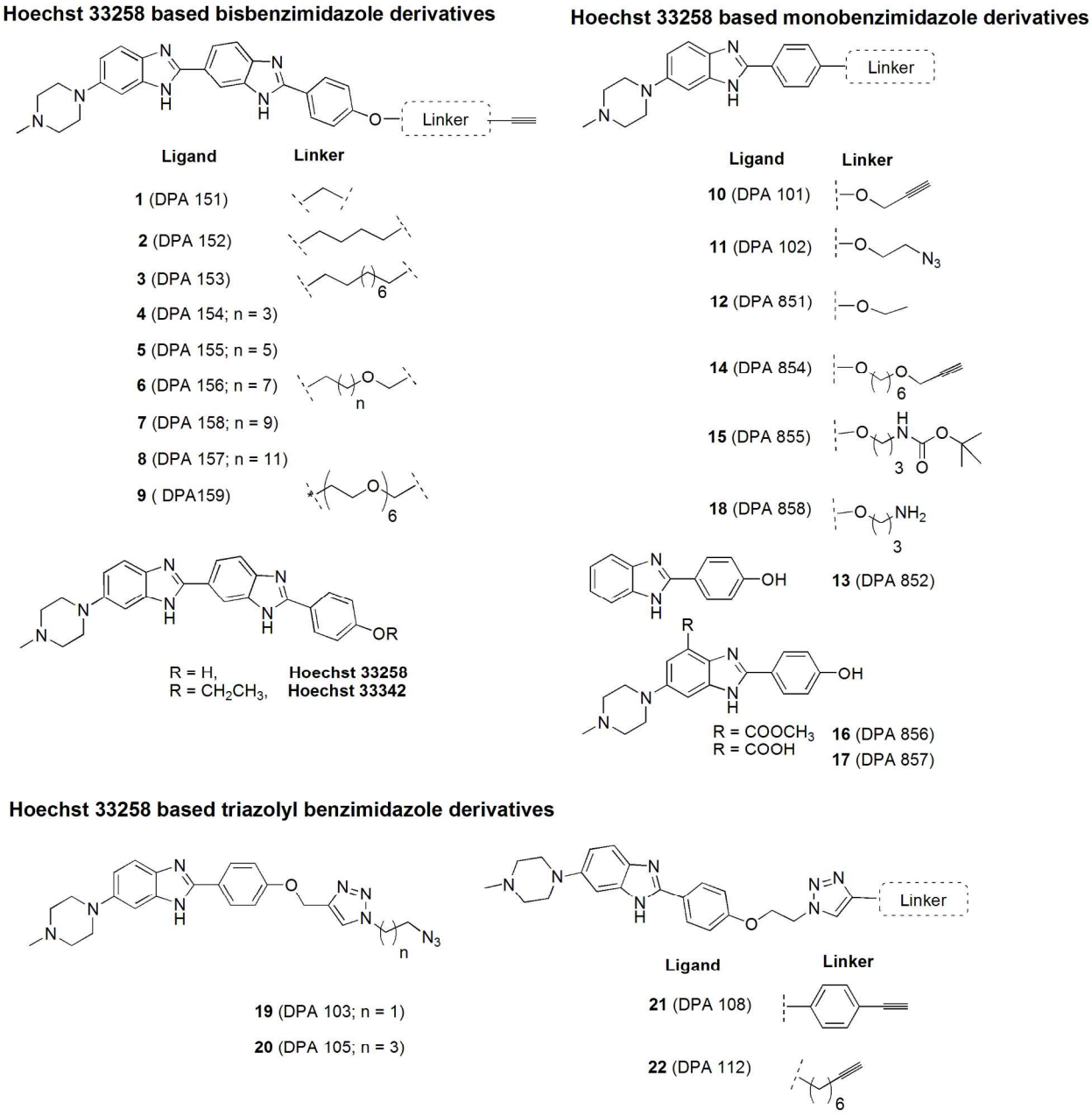


Figure 1. Chemical structures of compounds used in this study.

Of other features such as the presence of bulky groups on terminal phenyl rings, linkers containing long alkyl groups add to the DNA poisoning abilities of these compounds.[26, 35] We

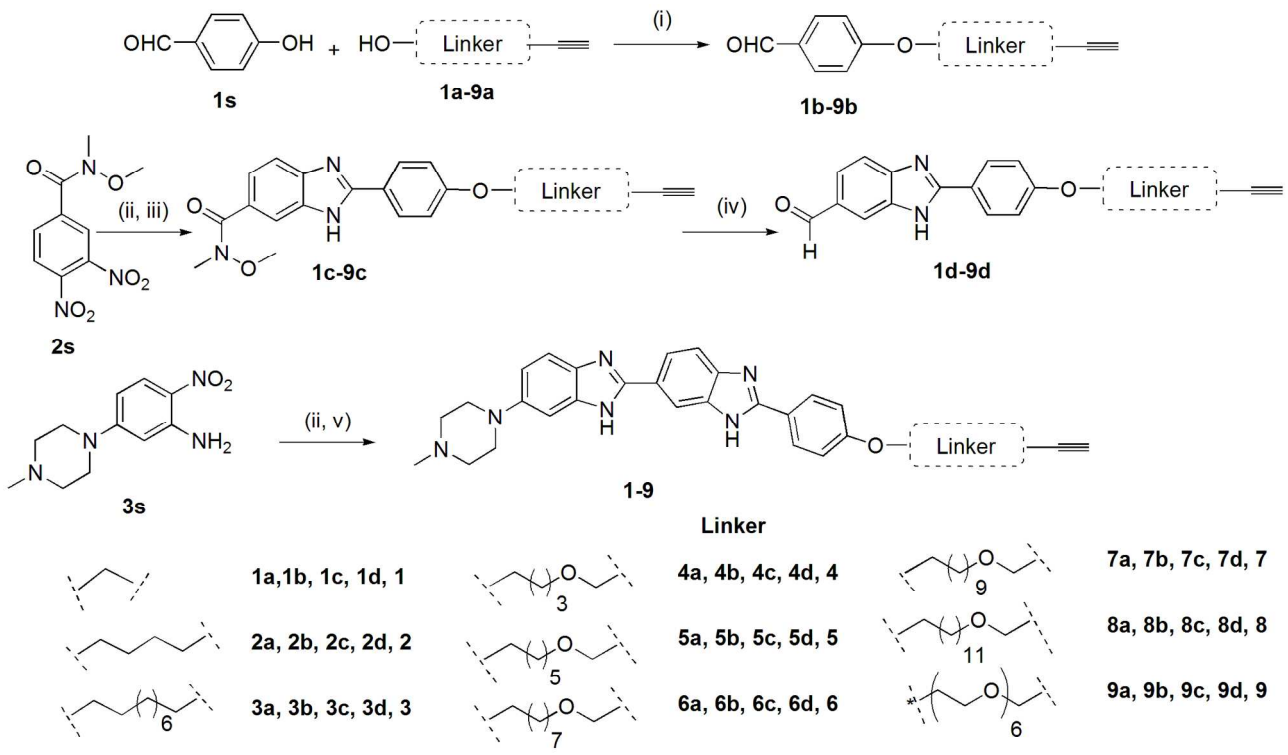
have recently reported that a Hoechst 33258 derived bisbenzimidazole with a linker containing a long alkyl group (twelve carbon atoms) showed excellent DNA topoisomerase I inhibition.[35]

In this article, we investigate the bacterial DNA and RNA topoisomerase inhibition of bisbenzimidazoles (Figure 1)[36] by systematically a] varying the alkyl chain length b] comparing the antibacterial and topoisomerase activity of bisbenzimdiazoles with mono-benzimidazoles derived from Hoechst 33258. We then explore the relationships between the topoisomerase I inhibition, antibacterial activity and DNA binding of these compounds.

RESULTS AND DISCUSSION

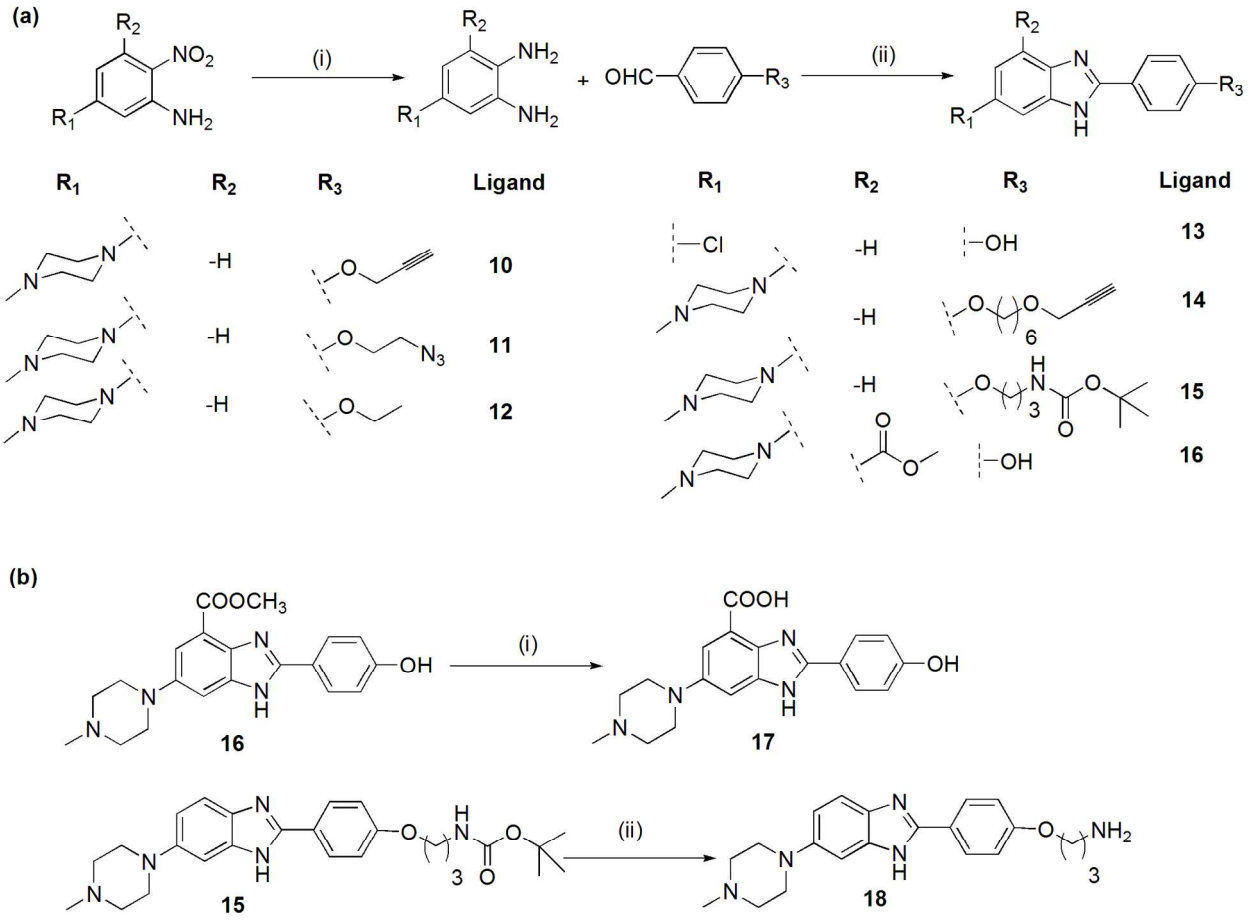
Synthesis of Novel Mono and Bisbenzimidazoles.

The alkynyl bisbenzimidazoles (**1-9**) were synthesized using literature procedures reported earlier.[35, 37-40] As shown in Scheme 1, a divergent strategy was employed. To introduce the linkers, we performed Mitsunobu reactions[41] of 4-hydroxy benzaldehyde with aliphatic alcohols (**1a-9a**) that terminated in an alkyne functionality (the aliphatic alcohols were obtained commercially or prepared in one step from corresponding diols).[42] This reaction afforded 4-substituted benzaldehydes (**1b-9b**) which were coupled with 3,4-diamino-N-methoxy-N-methylbenzamide in the presence of an oxidant to yield corresponding benzimidazoles (**1c-9c**) derivatives.[37, 38] The benzimidazoles (**1c-9c**) which contained Wienreb amide on one of the phenyl rings were easily reduced to corresponding aldehydes (**1d-9d**) using lithium aluminum hydride.[38] Coupling of aldehydes (**1d-9d**) with 4-(4-methylpiperazin-1-yl)benzene-1,2-diamine[43], in the presence of $Na_2S_2O_5$ resulted in the synthesis of alkynyl bisbenzimidazoles **1-9** in good yields (55-72%).



Scheme 1. Reagent and conditions (i) PPh_3, DIAD, dioxane, dichloromethane, rt, overnight, 50-85 %, (ii) Pd-C, H_2, ethanol,rt, 5h, qaunt, (iii) **1b-9b**, ethanol, $Na_2S_2O_5$, H_2O, reflux, 12-14 h, 61-85 % (for two steps), (iv) THF- ether, LAH , -78 ^oC to rt., 6-12 h, 42-73 %, (v) **1d-9d**, ethanol, $Na_2S_2O_5$, H_2O, reflux, overnight, 55-72 % (for two steps).

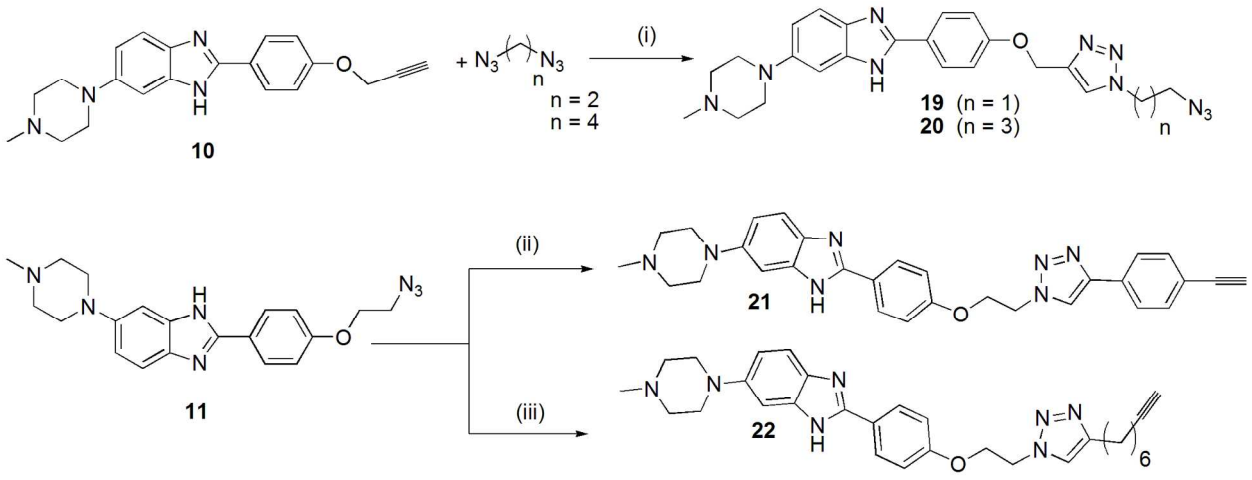
To achieve the synthesis of the monobenzimidazoles, three different strategies were employed. For the synthesis of monobenzimidazoles that bear no triazolyl functionalities (compounds **10-14**, **16**, **18**), the desired compounds were obtained via condensation of an ortho-diamine with an appropriate aldehyde[44, 45] (Scheme 2a) in the presence of an oxidant (sodium metabisulfite).[37]



Scheme 2. Reagent and conditions (a) (i) Pd-C, H_2, ethanol, rt, 5 h, quant. (ii) $Na_2S_2O_5$, H_2O, ethanol, reflux, overnight, 47-84 % (b) (i) 1N NaOH (aq), 80 ^oC, 58 % (ii) Trifluoroacetic acid (TFA), dichloromethane (DCM), rt, quant.

Monobenzimidazole derivative **15** was synthesized by the basic hydrolysis of **14**, while **17** was obtained by the removal of the Boc protecting group of **16** using trifluoroacetic acid (Scheme 2b). To achieve the synthesis of triazolyl containing monobenzimidazoles (**19-22**), we exploited the chemical nature of the functionalities present at the ends of **10** (a terminal alkyne) and **11** (an azide). Alkynes and azides are key components for the copper catalyzed 1, 3 dipolar cylcoaddition, which results in the regioselective formation of 1,4 substituted 1,2,3 triazoles.[46, 47] Compound **10** was reacted with excess bisazides to afford triazole bearing mono-benzimidazoles

that terminate with an azide end (**19, 20**). Similarly, compound **11** was reacted with excess bisalkynes to yield triazole bearing mono-benzimidazoles (**21, 22**) that terminate with an alkyne end (Scheme 3).[44,48]



Scheme 3. Reagent and conditions (i) ethanol, sodium ascorbate, $CuSO_4$, rt, 24 h, 30-68 % (ii) 1, 4 diethynyl benzene, ethanol, sodium ascorbate, $CuSO_4$, rt, 24 h, 57% (iii) 1,9 decadiyene, ethanol, sodium ascorbate, $CuSO_4$, 24 h, 79% .

Enhanced and Selective Inhibition of *E. coli* DNA Topoisomerase I. Enzymes, such as topoisomerase I and gyrase are potential targets of antibacterial compounds.[12, 49] Bisbenzimidazoles and their derivatives are well known to inhibit the activity of eukaryotic DNA topoisomerase I and promote anti helicase activity.[50] Related bisbenzimidazole derivatives have also been shown to poison eukaryotic DNA topoisomerase I activity which is believed to elicit its effect in a manner similar to camptothecin which involves DNA and protein binding.[33] To probe the role of terminal alkyl chain length, we have synthesized a small library of bisbenzimidazoles (**1-9**) where the length of terminal alkyl chain ranges from 3-21 atoms.

Additionally, to gauge the effect of bisbenzimidazole vs. monobenzimdiazole moiety, we have synthesized mono-benzimidazoles related to Hoechst 33258 (**10-22**) to compare their DNA topoisomerase and bacterial inhibition properties with bisbenzimidazoles (**1-9**). The inhibitory effects of the compounds (**5-22**) were tested against four DNA topoisomerases (*E. coli* DNA topoisomerase I, *E. coli* DNA gyrase, human DNA topoisomerase I, human DNA topoisomerase II).

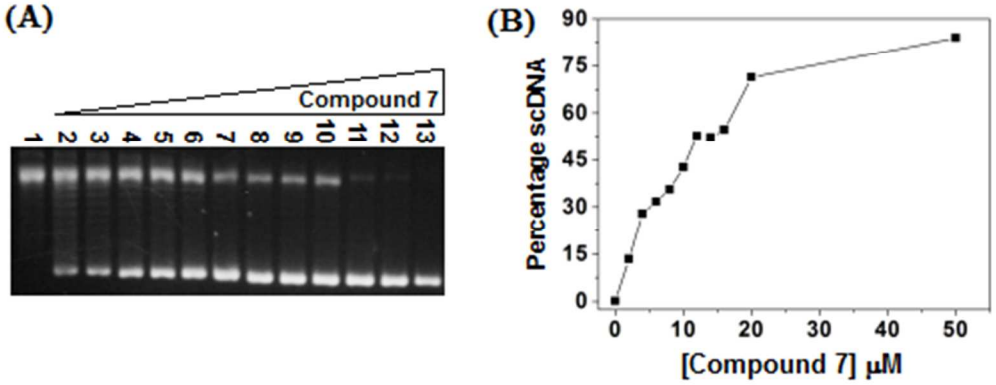


Figure 2. A representative image showing the inhibitory activity of bisbenzimidazole 7 against *E. coli* DNA topoisomerase I. (A) The inhibition effects of bisbenzimidazole 7 against *E. coli* DNA topoisomerase I. The *E. coli* DNA topoisomerase I inhibition assays were performed as described in the experimental section. The plasmid DNA was isolated and subjected to 1% agarose gel electrophoresis. Lanes 1 to 13 contain 0, 2, 4, 6 8, 10, 12, 14, 16, 18, 20, 30, and 50 µM of bisbenzimidazole 7 respectively. (B) The quantification analysis of the inhibitory activities of bisbenzimidazole 7 against *E. coli* DNA topoisomerase I. The values of IC_{50} were obtained from these analyses. Standard deviation was obtained from three independent determinations. (scDNA = Supercoiled DNA).

A representative inhibition of *E. coli* DNA topoisomerase I is displayed in Figure 2 (also see supporting information, Figures S37- S40). In this assay, the relaxation of supercoiled plasmid

pBAD-GFPuv was observed in the presence of various inhibitors. In the absence of inhibitor, *E. coli* DNA topoisomerase I fully relaxed the supercoiled plasmid DNA pBAD-GFPuv (Fig. 2A, lane 1). Lanes 2-13 show the inhibitory effect of compound **7** on the relaxation. As increasing amounts of compound **7** were added, the percentage of supercoiled DNA increased which attained saturation level at inhibitor concentration 20 μM and above. The IC_{50} values of effective topoisomerase inhibitors are summarized in Table 1. All mono-benzimidazoles, except compounds **9** and **20**, showed enhanced inhibition of *E. coli* DNA topoisomerase I. While compound **9** at 50 μM did not significantly inhibit the activity of *E. coli* DNA topoisomerase I, compound **20** showed an IC_{50} of 21.5 μM, which is comparable to the IC_{50} value of Hoechst 33258. We also tested the inhibitory effects of these compounds against *E. coli* DNA gyrase and found that none of the tested bisbenzimidazoles (**1-4**, **8**) showed any inhibitory activities against *E. coli* DNA gyrase. Nevertheless, results in Table 1 reveal that all of the bisbenzimidazoles except compounds **9** and **20** have much more enhanced inhibition (requiring <13 μM inhibitor) of *E. coli* DNA topoisomerase I than Hoechst 33258 or Hoechst 33342 does.

Table 1. IC_{50} values of the bisbenzimidazoles (**1-9**) and monobenzimidazole (**20**) against *E. coli* DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase II

	IC_{50} (μM)[a]	

Compound	ecTopoI[b]	hTopoI[b]	hTopoII[b]
Hoechst 33258 [c]	19.50±1.32	22.86±1.55	28.92±4.45
Hoechst 33342 [c]	29.83±2.75	>50μM	-
1[c]	5.50±0.50	25.41±2.20	51.6±2.5
2[c]	4.57±0.81	>50μM	-
3[c]	2.47±0.06	>50μM	-
4[c]	6.63±0.47	>50μM	-
5	3.20±0.26	>50μM	-
6	2.80±0.26	>50μM	-
7	12.24±0.63	>50μM	>50 μM
8	4.00±1.32	>50μM	-
9	>50	-	-
20	21.50±2.18	>50μM	-

[a]IC_{50} was determined as described in the experimental section. The values are the average of at least three independent determinations. [b]ecTopo I, hTopo I, and hTopo II represent *E. coli* DNA topoisomerase I, human DNA topoisomerase I, and human DNA topoisomerase II, respectively. [c]The values have been reproduced from reference 35.

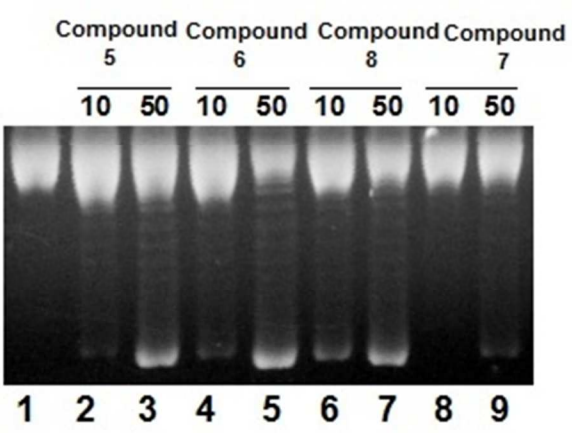


Figure 3. Decreased inhibitions of bisbenzimidazoles **5-8** against human DNA topoisomerase I. Inhibition assays against human topoisomerase I were performed as described in the experimental section in the presence of one of the bisbenzimidazoles. Following the inhibition assays, the plasmid DNA molecules were isolated and subjected to 1% agarose gel electrophoresis. Lane 1 represents the relaxed plasmid DNA pBAD-GFPuv. Lanes 2-8 represent the two different concentrations tested for each compound (10 and 50 μM) in these assays.

To determine their selectivity against *E. coli* DNA topoisomerase, we tested the inhibitory effects of bisbenzimidazoles **1-9** and the lone monobenzimidazole **20** (which had shown comparable *E. coli* DNA topoisomerase I IC_{50} values to Hoechst 33258) against human DNA topoisomerase I and II. **Figure 3** shows the human DNA topoisomerase I inhibition of compounds **5-8** at two tested concentrations 10 and 50 μM. Our results summarized in Table 1 clearly show the selectivity of bisbenzimidazoles towards the inhibition of bacterial DNA topoisomerase I. The IC_{50} values of these compounds against human topoisomerase I and II are much higher than that against *E. coli* topoisomerase I (Table 1).

Inhibition of RNA Topoisomerase: We and others have recently shown that *E.coli* topoisomerase I (EcoTop1) possesses not only topoisomerase activity for DNA, but also RNA.[51, 52] We, therefore, examined whether the compounds that inhibit the DNA topoisomerase activity

of EcoTop1, can also inhibit its RNA topoisomerase activity. We found that ethacridine and Hoechst 33342, which are known DNA binders and can inhibit DNA topoisomerase activity non-specifically, had little effect on the RNA topoisomerase activity of EcoTop1, even at 80 μM concentrations (Fig. 4A). In addition, six inhibitors **1, 2, 4, 5, 9** and **20** had no discernible inhibitory effect on the RNA topoisomerase activity of EcoTop1 at concentrations up to 50 μM (Fig. 4B). Notably, four inhibitors **3, 6, 7** and **8** inhibited the RNA topoisomerase activity at about 10 μM concentration. Our data thus suggest that only a selected group of inhibitors of DNA topoisomerase activity can also inhibit the RNA topoisomerase activity. The mechanism of why only these four inhibitors can inhibit the RNA topoisomerase activity remains to be investigated. However, close inspection of their chemical structure reveals that they all share one similarity: the long alkyl chain at their ends. Therefore, it is possible that these alkyl chains at the ends have a role in the RNA topoisomerase inhibition.

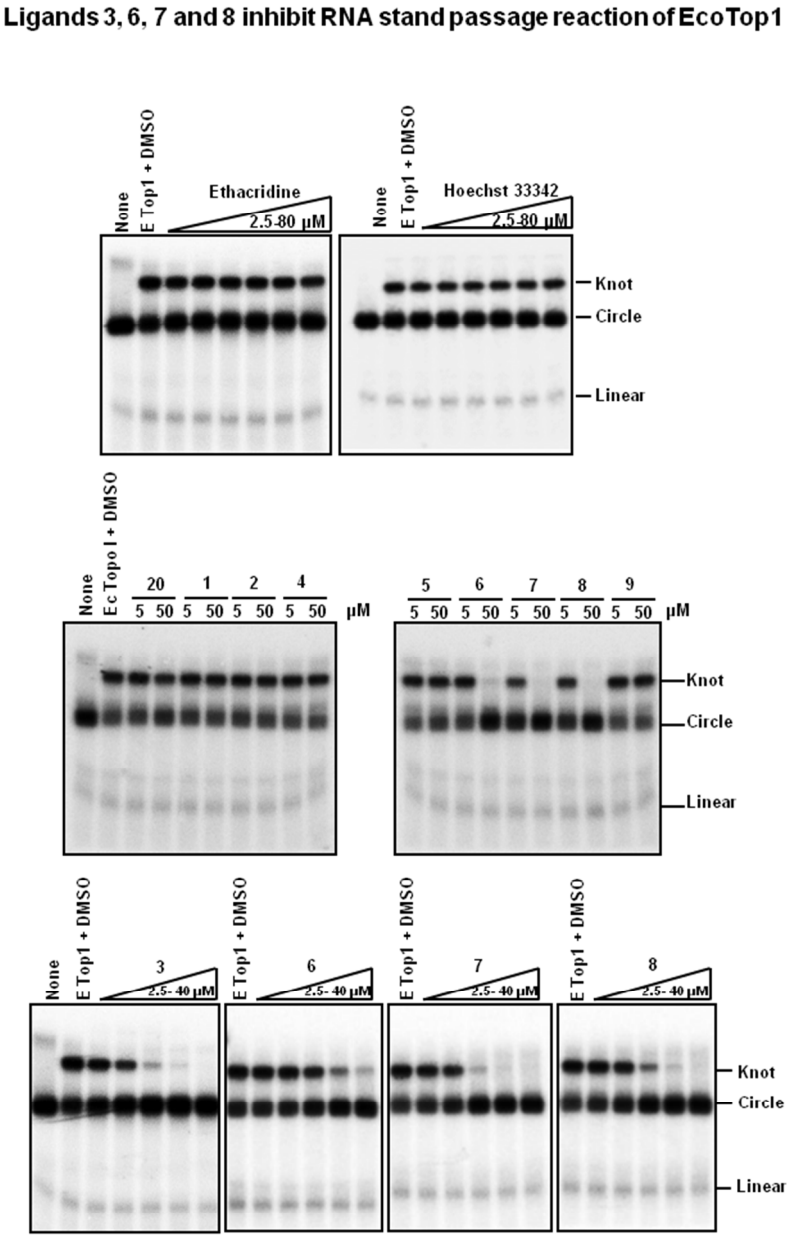


Figure 4: Effect of inhibitors towards inhibiting RNA topoisomerase activity of EcoTop1. (A) Autoradiographs show that ethacridine and Hoechst 33342 do not have any effect on RNA topoisomerase activity of EcoTop1. The inhibitor concentrations are 2.5, 5, 10, 20, 40 and 80 µM (lane 3-8) (B) Autoradiographs of inhibitors **1-9** and **20** show that out of ten inhibitors tested, only **3** , **6**, **7** and **8** inhibit RNA topoisomerase activity of EcoTop1 at 50 µM concentration

while others **1**, **2**, **4** , **5** and **20** did not inhibit RNA topoisomerase activity of EcoTop1 at 50 μM concentration (C) Autoradiographs show concentration-dependent inhibition of EcoTop1 RNA topoisomerase activity by inhibitors **3**, **6**, **7** and **8**. In all the reactions, 10 nM EcoTop1 was used. Since all inhibitors were diluted in DMSO, a control reaction (Lane 2 of every autoradiograph) with DMSO only also performed. The final inhibitor concentrations were 2.5, 5, 10, 20, and 40 μM (lane 3-7). The autoradiography was done using Phosphoimager (Molecular Dynamics).

UV thermal denaturation studies show the requirement of a bisbenzimidazole unit for B-DNA binding. DNA associated with topoisomerases plays an important role in the inhibitor's activities and usually forms a DNA-inhibitor-topoisomerase ternary complex. Therefore, the DNA binding studies could provide information about any relationship between inhibitor -DNA interaction and the topoisomerase inhibitor activity. Bisbenzimidazoles are known topoisomerase inhibitors whose inhibitory activity has been proposed to stem from ternary complex formation involving AT rich DNA sites, the topoisomerase enzyme and the inhibitor[32] as well as its binding at AT rich distal site on DNA.[53] These studies have revealed that such inhibition does not have strong DNA binding dependence and that their minor groove binding may not be the sole determinant of topoisomerase inhibition. Bisbenzimidazole Hoechst 33258 is an established AT rich B-DNA binder known to bind in the DNA minor groove with nanomolar affinities.[54-58]

Table 2. A table showing the thermal denaturation temperatures of duplex DNA ($dA_{60}.dT_{60}$) in the presence of all studied compounds (10 μM each) in buffer 10 mM sodium cacodylate, 0.1 mM EDTA and 100 mM NaCl at pH 7.0.

Compound	T_m(°C)	ΔT_m (°C)
None	62.5	-
Hoechst 33258[a]	87.1	24.6
Hoechst 33342 [a]	86.6	24.1
1 [a]	85.4	22.9
2 [a]	83.4	20.9
3 [a]	70.5	8.0
4 [a]	85.9	23.4
5	80.8	18.3
6	75.6	13.1
7	68.1	5.6
8	63.7	1.2
9	75.6	13.1
10	65.1	2.6
11	64.4	1.9
12	64.2	1.7
13	62.5	0.0
14	63.9	1.4
15	63.5	1.0
16	62.5	0.0
17	62.5	0.0
18	65.8	3.3

19	63.7	1.2
20	63.0	0.5
21	64.4	1.9
22	63.0	0.5

[a]The values have been taken from reference 35.

To further evaluate the DNA binding ability of these compounds, we performed thermal denaturation studies with a B-DNA duplex $dA_{60}.dT_{60}$. The formation of the duplex was confirmed by the sharp melting transition at 62.5 ^{o}C in the presence of 100 mM NaCl. The compounds (10 μM) were mixed with the DNA (1 μM/duplex) and heated further to determine the thermal stability. At this ratio, the DNA was saturated with the compound as using lower concentrations (< 5 μM) of the compound resulted in biphasic transitions (data not shown). The results obtained from the thermal denaturation studies are shown in Table 2 (for melting profiles see supporting information, Figure S36). Of the bisbenzimidazoles studied (**1-9**), few compounds (**1, 2, 5, 6**) displayed thermal stabilization greater than 18.0 ^{o}C which were comparable to the change in melting temperature afforded by Hoechst 33258 and 33242. However, compounds **3**, **7** and **8** showed much lower thermal stabilization (8.0 ^{o}C, 5.6 ^{o}C, and 1.2 ^{o}C respectively). The thermal stabilization afforded by bisbenzimidazole **8** is comparable to the thermal stabilization obtained with mono-benzimidazoles (**10-18**) and triazole bearing mono-benzimidazoles (**19-22**). The differential thermal stabilization by bisbenzimidazoles indicates the role of linker length in perturbing the DNA minor groove binding of the benzimidazole moeity.

All mono-benzimidazoles (**10-18**) showed only up to a 3 ^{o}C increase in the thermal stabilization of $dA_{60}.dT_{60}$ duplex. Similar thermal stabilization was also found with the triazolyl-benzimidazoles (**19-22**). The thermal stabilization afforded by mono-benzimidazoles was much smaller in comparison to most of the bisbenzimidazoles which showed up to ~ 25 ^{o}C increases in the thermal denaturation temperatures of $dA_{60}.dT_{60}$ duplex. These results confirm that mono-benzimidazoles are much weaker DNA binders than bisbenzimidazoles. Wilson and coworkers have previously shown that Hoechst 33258 like compounds with two benzimidazoles units (but lack the piperazine unit) afford similar thermal stabilizations as Hoechst 33258 when bound in the minor groove of B-DNA.[22] However, Hoechst 33258 derived mono-benzimidazoles, which terminate with a guanidinium group or an imidazole (in place of piperazine), can produce much higher thermal stabilization in comparison with compounds containing a piperazine.[22] This observation, coupled with our findings, suggest that key requirements of the Hoechst 33258 derived compounds for DNA binding are the presence of a linked bisbenzimidazole skeleton and moieties capable of making significant electrostatic interactions. Our data presented in Table 2 similarly highlight the requirement of a bisbenzimidazole moiety for effective B-DNA recognition. These results are important in the context of converting these DNA binding compounds into enzyme inhibitors with reduced background genotoxicity. Our data clearly show that with appropriate modifications, DNA binding and topoisomerase enzyme inhibition activities can be decoupled.

Antibacterial Studies. Compounds belonging to the bisbenzimidazole class have shown significant antibacterial effect against a variety of strains, which include methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Entercoccus faecalis*.[59, 60] Hoechst

33258 has been reported to have antimicrobial activity against *Pneumocystis carinii*f. sp. *muris*, *Candida albicans* and *Candida dubliniensis*.[61]We have probed the antibacterial activity of all compounds described in the study. The antibacterial effect evaluation of compounds (**1-22**) included both gram positive and gram negative strains (Table 3). In cases where a sharp inflection in the bacterial growth was not observed, the MIC is given as a range of values.

Nearly all mono-benzimidazoles (**10-22**) displayed similar MIC values (16-32 μg/mL) against gram positive bacterial strains *S. aureus* ATCC 29213, *S. aureus* ATCC 33591, S. *epidermidis*, the two MRSA strains, *E. faecium*, and *E. faecalis*. A number of bisbenzimidazoles (Hoechst 33258, Hoechst 33342, **1**, **2**, and **4**) showed much lower MIC values against most of the strains (0.3 - 4 μg/mL). Bisbenzimidazoles **3** and **5-9** showed relatively higher MIC values (16-32 μg/mL respectively) than other bisbenzimidazoles. As described in the aforementioned sections, **3** and **5-9** also showed poor/decreased DNA binding as compared to Hoechst 33258 as evident from thermal denaturation experiments. Bisbenzimidazoles **3** and **5-9** have a common feature in their chemical structure, which is the presence of long alkyl linker. The presence of the long aliphatic group at the terminal renders the molecule to be more hydrophobic. The alkyl chains can impact the B-DNA binding of these molecules possibly due to self-aggregation of alkyl groups in aqueous solutions. It is unclear if the alkyl chain length play a similar role in affecting the antibacterial activity of these compounds.

Table 3. Minimal inhibitory concentrations (MIC) of the studied compounds against various bacterial strains by microbroth dilution.

	MIC (μg/mL)				
Compound	MRSA 33591	MRSA A960649	*S. aureus* 29213	*S. aureus* 33591	*S. epidermidis* 12384

Hoechst 33342	1	0.5	2-4	2-4	1
Hoechst 33258	ND	ND	≥32	16-32	ND
1	8	1-2	2-4	2-4	1-2
2	4	4	2-4	2-4	1-2
3	>32	>32	16	16	>32
4	1	1-2	2-4	2-4	4
5	>32	>32	16-32	16-32	>32
6	>32	>32	>32	16-32	>32
7	>32	>32	>32	>32	>32
8	>32	>32	16-32	16-32	>32
9	>32	>32	16-32	16-32	16-32
10	>32	>32	≥32	16-32	>32
11	>32	>32	≥32	16-32	>32
12	>32	>32	16-32	16-32	>32
13	>32	>32	16-32	16-32	>32
14	>32	>32	16-32	16-32	>32
15	>32	>32	16-32	16-32	>32
16	>32	>32	16-32	16-32	>32
17	ND	ND	>32	>32	ND
18	>32	>32	>32	>32	>32
19	>32	>32	16-32	16-32	>32
20	>32	>32	16-32	16	>32
21	>32	>32	16-32	16	>32
22	>32	>32	16-32	16-32	>32

MIC (μg/mL) continued

Compound	*E. faecium* BM4105RF	*E. faecalis* 29212	*S. flexneri* 2457NR517	*E. coli* 25922	*E. coli* K12
Hoechst 33342	0.3	16-32	1-2	16	16
Hoechst 33258	ND	16-32	ND	8-16	8
1	1-2	16-32	1-2	8	8
2	1-2	4	8	8-16	8-16
3	>32	16-32	>32	16	8-16
4	1-2	8	4	8-16	8-16
5	>32	16-32	>32	16-32	8
6	>32	16-32	>38	16-32	2-4
7	>32	16-32	>32	>32	2-4
8	≥32	16-32	>32	16	16
9	≥32	16-32	≥32	16-32	2-4
10	>32	16-32	>32	16	16
11	>32	16-32	>32	16	16

12	>32	16	>32	16	16
13	>32	16	>32	16	16
14	>32	16-32	>32	16	16
15	>32	16-32	>32	16	16
16	>32	16-32	>32	16	16
17	ND	>32	ND	>32	>32
18	>32	>32	>32	>32	>32
19	>32	16-32	>32	16	16
20	>32	16-32	>32	16	16
21	>32	16-32	>32	16	16
22	>32	16-32	>32	16	16

MIC (μg/mL) continued

Compound	*A. baumanni* 19606	*P. aeruginosa* 27853	*K. pneumoniae* NR15410	*E. cloacae* 13047	*C. freundii* 4747CFAA
Hoechst 33342	8	16	8	4	8
Hoechst 33258	ND	8	ND	ND	ND
1	>32	16	>32	32	4
2	16	16	>32*	>32	>32*
3	>32	16	>32	>32	>32
4	8	16	>32*	>32*	16
5	>32	16-32	>32	>32	>32
6	>32	16-32	>32*	>32	>32
7	>32	16-32	>32	>32	>32
8	>32	16	>32	>32	>32
9	>32	16-32	>32	>32	>32
10	>32	16	>32	>32	>32
11	>32	16	>32	>32	>32
12	>32	16	>32	>32	>32
13	16-32	16	>32	>32	>32
14	>32	16	>32	>32	>32
15	>32	16	>32	>32	>32
16	>32	16	>32	>32	>32
17	ND	>32	ND	ND	ND
18	>32	>32	>32	>32	>32
19	>32	16	>32	>32	>32
20	>32	16	>32	>32	>32
21	>32	16	>32	>32	>32
22	>32	16	>32	>32	>32

*Indicates the %inhibition in *K. pneumoniae*, *E. cloacae* and *C. freundii* was the greatest for these compounds (though with low values) as compared to the other compounds.

Bisbenzimidazoles (**1-6** and **8**) that effectively inhibit *E. coli* DNA topoisomerase I and RNA topoisomerase (**3, 6, 7, 8**) also display varying degrees of antibacterial activity, with compound **4** being the most potent overall. For gram negative strain *Escherichia coli* K 12, long alkyl chains on some bisbenzimidazoles imparted better antibacterial activity. Typically all of the bisbenzimidazole compounds that had shorter alkyl linkers displayed better antibacterial activity than same compound with alkyl ends greater than ten atoms. Overall, these results show that alkynyl bisbenzimidazoles derived from parent Hoechst 33258 are capable of inhibiting both gram positive and gram negative bacteria.

Cytotoxicity and Docking Studies.

Cytotoxicity of compounds **1-9** was determined against human prostate cancer cell line DU-145. Our results indicate that some of these compounds (**1**, **2**, **4**, **5**, **6**) are more cytotoxic to DU-145 than parent compound Hoechst 33258 (IC_{50} >10 μM) while others (compounds **3**, **7**, **8**, **9**) display comparable inhibitory effects (IC_{50} >10 μM) to Hoechst 33258 (Table S1, see supporting information). A feature clearly discernible among compounds **1**, **2**, **4-6** (linker length up to 12 atoms**)** and **3**, **7-9** (linker length up to 21 atoms) is the linker length dependent cytotoxicity of these compounds. Revisiting the thermal stability results shown in table 3, it can be observed that compounds **3**, **7-9** display poorer binding to B-DNA than compounds **1**, **2**, **4-6**. These results indicate that the cytotoxicity of these compounds may have its origins in their DNA binding abilities. One of the best DNA and RNA topoisomerase inhibitor (compound **8**) also showed similarly low cytotoxicity to a normal dermal fibroblast cell line PCS 201-010 with IC_{50} >10 μM.

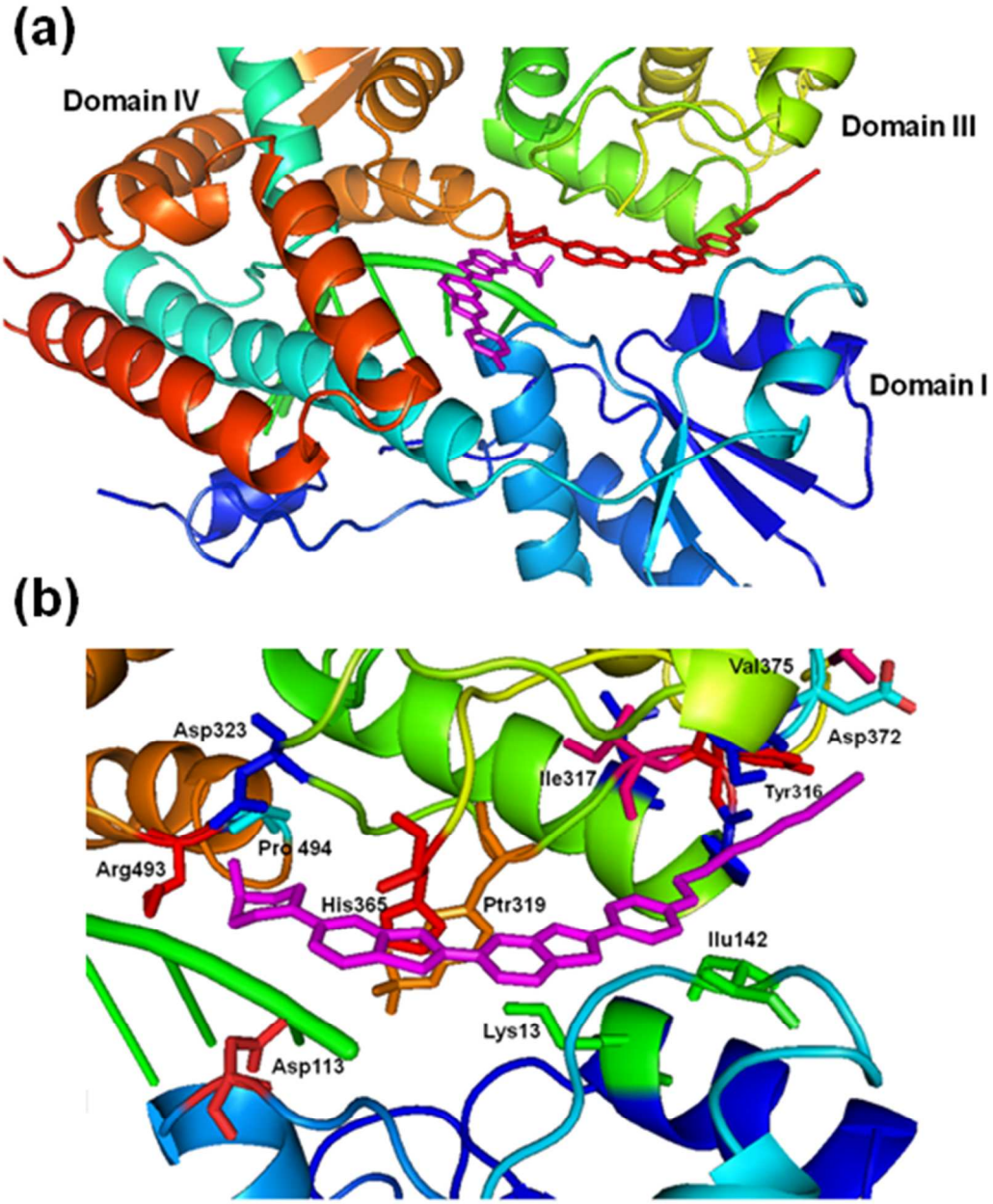


Figure 5. (a) Docked pose of Hoechst 33258 (red) and compound **3** (magenta) at the active site of *E. coli.* topoisomerase I covalent complex showing differences in the bisbenzimidazole unit orientation of the two compounds. (b) A closer view compound **3** (magenta) bound at the active site showing the amino acid residues that are involved in the ternary interaction.

We have performed molecular docking experiments to gain insight into the inhibitor binding to bacterial topoisomerase I. We (Tse-Dinh) have previously reported the crystal structures of the active site of *E. coli.* topoisomerase I including a structure where the covalent intermediate is trapped with a cleaved single stranded oligonucleotide substrate.[62] Using the structure of this enzyme-DNA complex (PDB ID: 3PX7), we performed docking experiments with one of the most potent *E. coli.* topoisomerase I inhibitor: compound **3**. As a control experiment, we also docked Hoechst 33258 to observe any spatial differences in their binding at the active site. Docking of compound **3** showed that the benzimidazole moiety is in the vicinity of the cleaved oligonucleotide at the active site with the piperazine end being closest to it. Compound **3** makes several interactions (hydrogen binding, π and van der Walls) at the active site, with additional interactions with amino acids Arg493, Asp323, Pro494 residues. Both benzimidazole moieties of compound **3** make interactions with the amino acid residues. The benzimidazole unit adjacent to the piperazine unit makes π-interactions with Asp113 while the other benzimidazole unit close to the phenoxy group makes π and van der Walls interactions with Lys113 and Ptr319 residues. The long alkyl chain is directed away from the active site towards helices J and K of domain III[63] of the enzyme where it makes key contacts with Val375, Asp372 and Tyr316. Contrary to this, the Hoechst 33258 docking results showed a somewhat different view with major difference being that the piperazine end of the molecule protrudes away from the active site in the proximity of helices A and D of domain I and helix Q of domain IV. Overall, these results suggest that the additional hydrophobic interactions made by the alkyl chain of compound **3** with helices J and K of domain III provide critical interactions for binding of the inhibitor, leading to a stronger and more selective inhibition than Hoechst 33258. For additional details of docking studies, please see appendix I in the supporting information.

CONCLUSIONS

Benzimidazoles derived from Hoechst 33258 are an important class of molecules that are effective against wide range of biological targets. Since molecules belonging to bisbenzimidazole class are known inhibitors of mammalian topoisomerases as well, they have been widely used in targeting cancer growth. The results obtained from this study show that Hoechst 33258 derived alkynyl bisbenzimidazoles are much more effective *E. coli* topoisomerase I inhibitors than mono-benzimidazoles derivatives and are much more selective in their inhibition properties when compared to Human topoisomerases I and II. The DNA binding ability and topoisomerase I inhibitions of these compounds are unrelated suggesting that novel compounds that inhibit these enzymes but show reduced binding to background DNA can be identified using our approach. Since some bisbenzimidazoles with longer alkynyl linkers showed improved IC_{50} values than the compounds with shorter linkers, it is plausible that alkyl linkers interact with the enzyme leading to greater disruption of the DNA-topoisomerase I complex. Our modeling results lend credence to such an interaction between the enzyme and the ligand side chain. There is an increasing interest in developing small molecules that selectively target bacterial topoisomerases over mammalian topoisomerases. These compounds present a new structural feature (long alkyl ends terminating in an alkyne) that can lead to selective inhibition of bacterial topoisomerase I over human topoisomerases I and II as well. The same structural feature also appears to be responsible for its RNA topoisomerase inhibition activity. Structural studies are needed to gain a deeper insight in the interaction of these bisbenzimidazoles with topoisomerases, and to better elucidate the preferential binding site of long alkyl chains.

EXPERIMENTAL SECTION

General Methods. Chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and used as obtained from the supplier. Hoechst 33258 and Hoechst 33342 were obtained as their hydrochloride salts and used without further purification. Di-*tert*-butyl dicarbonate (Boc anhydride) was purchased from Advanced Chem Tech (Louisville, KY). All solvents were purchased from VWR (Atlanta, GA). Silica gel (32-65 μM mesh size) was purchased from Sorbtech (Atlanta, GA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance (300/500 MHz) spectrometer. Chemical shift are given in ppm and are referenced to residual solvent peaks (^{1}H and ^{13}C NMR). Mass (MALDI-TOF) spectra were collected using a Bruker Microflex mass spectrometer. Ultra Violet (UV) spectra were collected on a Varian (Walnut Creek CA) Cary 100 Bio UV-Vis spectrophotometer equipped with a thermoelectrically controlled 12-cell holder. HPLC analysis of all new compounds reported in this article was done on HP1100 series analytical HPLC instrument. The purity of all tested compounds was > 95%. The autoradiography was done using Phosphoimager (Molecular Dynamics).

Nucleic Acids. Nucleic acids were purchased from Eurofins MWG Operon (Huntsville, AL). The nucleic acids were used without further purification and their concentration was determined using the extinction coefficients provided by the supplier. Nucleic acid solutions were prepared in buffer 10 mM sodium cacodylate, 0.1 mM EDTA and 100 mM NaCl at pH 7.0 by heating at 95 ^{o}C for 15 minutes and then slowly allowing it to cool back to room temperature. After two days of incubation, the duplex formation was checked by UV thermal denaturation experiments. The stock solution was stored at 4 ^{o}C and diluted to desired concentrations as required.

Synthesis of compounds **1b-4b, 1c-4c, 1d-4d, 4a, 1-4**, **10**, **11** and **20** have been reported elsewhere.[35,48]

6-(prop-2-ynyloxy) hexan-1-ol (5a). To a solution of 1, 6 hexanediol (7.00 g, 59.2 mmol) in dry THF (60.0 mL), sodium hydride (1.08 g, 45.0 mmol) was added under argon at 0 ^{o}C and stirred for 30 min.[42] To this, propargyl bromide (5.00 g, 42.0 mmol) was added and stirred at 0 ^{o}C for another 30 minutes. The mixture was allowed to warm to room temperature and stirred for 24 h. The progress of reaction was monitored using TLC (silica gel). The reaction was quenched by pouring the reaction mixture into water. The mixture was extracted with diethyl ether (3 × 100 mL). The organic layers were combined and dried with sodium sulfate. The volatiles were evaporated and the crude product was purified by column chromatography on a silica gel column using hexanes–ethyl acetate as eluent to afford the desired product as pale yellow oil (2.80 g, 42 %): R_f = 0.31 (hexanes-ethyl acetate 7:3 *v/v*); IR (neat, cm^{-1}) 3399 (-OH H-bonded), 3297 (alkyne C-H stretch), 2937 (aromatic C-H stretch), 2868, 2120 (alkyne C-C stretch), 1711, 1462, 1360, 1241, 1090, 669;^{1}H NMR (300 MHz, $CDCl_3$) δ 4.07 (d, *J* = 2.3 Hz, 2H, -CH_2OCH_2CCH), 3.54 (t, *J* = 6.7 Hz, 2H, -OCH_2CH_2-), 3.46 (t, *J* = 6.6 Hz, 2H), 2.56 (br, 1H), 2.41 (t, *J* = 2.3 Hz, 1H, -CH_2OCH_2CCH), 1.58-1.48 (m, 4H), 1.35-1.28 (br, m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 79.8, 74.2, 70.0, 62.4, 57.9, 32.5, 29.3, 25.8, 25.5.

4-(6-(prop-2-ynyloxy)hexyloxy) benzaldehyde (5b). To a solution of *p*-hydroxybenzaldehyde (1.50 g, 12.2 mmol) in dry dichloromethane-dioxane (30.0 mL, dichloromethane: dioxane 4:1, *v/v*), triphenyl phosphine (4.75 g, 18.2 mmol) and 6-(prop-2-ynyloxy) hexan-1-ol (1.87 g, 12.2 mmol) were added and the solution was ice cooled. To this, diisopropylazodicarboxylate (DIAD) (3.66 g, 18.2 mmol) was added at 0 ^{o}C slowly over 15 min. The contents were initially stirred at 0 ^{o}C for 30 min and then at room temperature overnight. Progress of the reaction was monitored

by thin layer chromatography on silica gel. The solvents were removed under reduced pressure. The gummy residue was dissolved in 100 mL (1:1 *v/v*) ethyl acetate:hexanes and was allowed to stand in the refrigerator for a day. The precipitated solid was filtered off and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes-ethyl acetate as eluent which afforded the desired compound as pale yellow liquid (1.10 g, 32 %): R_f = 0.73 (hexanes: ethyl acetate 6.5:3.5, *v/v*); IR (neat, cm^{-1}) 3288 (alkyne C-H stretch), 2945 (aromatic C-H stretch), 2859, 2160 (alkyne C-C stretch), 2060, 1683, 1593, 1503, 1315, 1254, 1164, 1106, 1025, 841, 661, 620; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.88 (s, 1H), 7.83 (d, *J* = 8.77 Hz, 2H), 6.99 (d, *J* = 8.77 Hz, 2H), 4.15 (d, *J* = 2.29 Hz, 2H, -CH_2OCH_2CCH), 4.05 (t, *J* = 6.30 Hz, 2H, -OCH_2CH_2-), 3.54 (t, *J* = 6.67 Hz, 2H), 2.44 (t, *J* = 2.48 Hz, 1H, -CH_2OCH_2CCH), 1.86-1.81 (m, 2H), 1.68-1.62 (m, 2H), 1.54-1.43 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.7, 164.2, 131.9, 129.8, 114.7, 80.0, 74.1, 70.0, 68.2, 58.0, 29.4, 29.9, 25.8, 25.7; MS MALDI-TOF *m/z* calcd for $C_{16}H_{20}O_3$ 260.32, found 261.87 $[M+H]^+$.

N-methoxy-N-methyl-2-(4-(6-(prop-2-ynyloxy)hexyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (5c). To solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.93 g, 3.65 mmol) in ethanol-ethyl acetate mixture (40.0 mL, 3:1 *v/v*), 10% Pd-C (0.30 g) was added. Hydrogenation for 5 h at atmospheric pressure yielded corresponding diamine which was, after filtration of the catalyst, used without further characterization (R_f = 0.46 in dichloromethane:methanol 9:1, *v/v*). 4-(6-(prop-2-ynyloxy) hexyloxy) benzaldehyde (0.95 g, 3.65 mmol) and sodium metabisulfite (0.69 g, 3.65 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 6 h. Volatiles were evaporated under reduced pressure. The crude product was purified on a silica gel column using dichloromethane-

methanol (0-10 % methanol in dichloromethane) as eluent to afford the desired product as thick brown liquid (1.15 g,73 %): R_f = 0.56 (in dichloromethane-methanol 9:1 *v/v*); IR (neat, cm^{-1}) 3327 (alkyne C-H stretch), 2933 (aromatic C-H stretch), 2859, 2180 (alkyne C-H stretch), 1617, 1491, 1254, 1172, 1094, 1021, 849, 743, 661; ^{1}H NMR (500 MHz, acetone-d_6) δ 12.23 (br, 1H), 8.18 (dd, J_1= 8.98 Hz, J_2 = 2.05 Hz, 2H), 7.97 (br, 1H), 7.59 (br, 2H), 7.07 (d, J_1 = 8.84, J_2 = 1.83, 2H), 4.14 (d, *J* = 2.15 Hz, 2H, -CH_2OCH_2CCH), 4.06 (t, *J* = 6.47 Hz, 2H, -OCH_2CH_2-), 3.61 (s, 3H), 3.52 (t, *J* = 6.68 Hz, 2H), 3.35 (s, 3H), 2.95 (t, *J* = 2.37 Hz, 1H, -CH_2OCH_2CCH), 1.83-1.77 (m, 2H), 1.64-1.58 (m, 2H), 1.54-1.41 (m, 4H) ; ^{13}C NMR (125 MHz, acetone-d_6) δ 170.1, 161.0, 153.4, 131.6, 128.3, 128.2, 122.7, 122.5, 114.8, 114.1, 80.3, 74.6, 71.1, 69.4, 67.9, 67.8, 60.2, 57.4, 33.3, 29.4, 25.7, 25.6; MS (MALDI-TOF) *m/z*calcd for $C_{25}H_{29}N_3O_4$ $[M]^+$ 435.21, found 436.57 $[M+H]^+$.

2-(4-(6-(prop-2-ynyloxy)hexyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (5d). To a solution of *N*-methoxy-*N*-methyl-2-(4-(6-(prop-2-ynyloxy)hexyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**5c**) (1.00 g, 2.37 mmol) in THF-ether (80.0 mL, 3:1), lithium aluminum hydride (0.26 g, 6.93 mmol) was added at -78 ^{o}C under argon and then allowed to stir at 0 ^{o}C for 8 h . The reaction mixture was quenched by the addition of saturated ammonium chloride solution (50.0 mL). The resulting grey precipitate was filtered off. The filtrate was extracted with ethyl acetate (3 × 50 mL). Organic layers were combined and dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using hexanes-ethyl acetate (0-80 % ethyl acetate in hexanes) as eluent to give the desired compound as sticky light yellow solid (0.36 g, 42 %): R_f = 0.51 (ethyl acetate-hexanes 8:2 *v/v*); IR (neat, cm^{-1}) 2929 (aromatic C-H stretch), 2855, 1687 (C=O stretch), 1605, 1262, 1184, 1094, 1012, 824, 743;^{1}H NMR (500 MHz, methanol-d_4) δ 10.04 (s, 1H), 8.13

(s, br, 1H), 8.06 (dd, J_1 = 8.83 Hz, J_2 = 1.99 Hz), 2H), 7.84 (dd, J_1 = 8.35 Hz, J_2 = 1.29 Hz, 1H), 7.72 (s, br, 1H), 7.10 (dd, J_1 = 8.81 Hz, J_2 = 2.01 Hz, 2H) , 4.16 (d, J = 2.27 Hz, 2H, -CH_2OCH_2CCH), 4.07 (t, J = 6.38 Hz, 2H, , -OCH_2CH_2-), 3.56 (t, J = 6.46 Hz, 2H) 2.85 (t, J = 2.26 Hz, 1H, -CH_2OCH_2CCH), 1.85-1.81 (m, 2H), 1.67-1.62 (m, 2H), 1.58-1.45(m, 4H); ^{13}C NMR (75 MHz, methanol-d_4) δ 192.4, 171.5, 161.5, 155.2, 139.5, 131.7, 128.3, 127.9, 123.7, 120.9, 115.9, 114.6, 79.4, 74.2, 69.5, 67.7, 57.3, 29.0, 28.8, 25.5, 25.4; MS (MALDI-TOF) *m/z* for calcd for $C_{23}H_{24}N_2O_3$ 376.17, found 377.59 ($[M+H]^+$) .

6-(4-methylpiperazin-1-yl)-2'-(4-(6-(prop-2-ynyloxy) hexyloxy) phenyl)-1H, 3'H-2, 5'-bibenzo[d]imidazole (5). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.20 g, 0.87 mmol) in ethanol- ethyl acetate mixture (20.0 mL, 3:1 *v/v*), Pd-C (0.10 g) was added followed by hydrogenation for 5 h at the atmospheric pressure. Catalyst was filtered off. To this solution, 2-(4-(6-(prop-2-ynyloxy) hexyloxy) phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**5d**) (0.33 g, 0.87 mmol) and a solution of $Na_2S_2O_5$ (0.16 g, 0.87 mmol) in water (1.00 mL) was added and the mixture was refluxed overnight. The reaction mixture was brought to room temperature. Volatiles were removed reduced pressure. The crude mixture was purified by column chromatography on a silica gel column using dichloromethane-methanol as eluent to afford the desired product as yellow solid (0.27 g, 56 %): R_f = 0.44 (dichlormethane-methanol 8:2 *v/v*);mp 178-180 ºC;IR (neat, cm^{-1}) 3390 (alkyne C-H stretch), 2942 (aromatic C-H stretch), 2853, 2259 (alkyne C-C stretch), 2128, 1611, 1452, 1256, 1175, 1020, 989, 816;^{1}H NMR (300 MHz, methanol-d_4) δ 8.21 (br, 1H) 7.99-7.91 (m, 3H), 7.65 (d, J = 8.56 Hz, 1H), 7.50 (d, J = 8.65 Hz, 1H), 7.12 (d, J = 1.79 Hz, 1H) 7.05-6.99 (m, 3H), 4.13 (d, J = 2.31 Hz , 2H), 3.94 (t, J = 6.48 Hz, 2H), 3.52 (t, J = 6.33 Hz, 2H), 3.22 (t, J = 4.64 Hz, 4H), 2.84 (t, J = 2.42 Hz, 1H), 2.71 (t, J = 4.71 Hz, 4H), 2.41 (s, 3H), 1.77-1.70 (m, 2H), 1.64-1.55 (m, 2H), 1.54-1.36 (m, 4H);

^{13}C NMR (75 MHz, DMSO-d_6) δ 160.7, 153.2, 147.9, 135.9, 132.5, 132.0, 129.3, 129.1, 128.7, 124.9, 122.7, 121.5, 120.8, 116.5, 115.3, 114.1, 109.2, 81.6, 77.3, 69.5, 68.1, 57.7, 55.1, 50.1, 45.8, 31.1, 29.3, 29.0, 25.8, 25.7; ESI-HRMS *m/z* cald for $C_{34}H_{39}N_6O_2$ 562.3131, found 562.3134 $[M]^+$; HPLC: t_R3.49 min, purity 97.8%.

8-(prop-2-ynyloxy) octan-1-ol (6a).To a solution of 1, 8 octanediol (8.00 g, 54.7 mmol) in dry THF (60.0 mL), sodium hydride (1.08 g, 45.0 mmol) was added under argon at 0 ^oC. The mixture was stirred for 30 min at 0 ^oC. To this, propargyl bromide (4.00 g, 35.0 mmol) was added and stirred at 0 ^oC for another 30 min. The reaction mixture was allowed to warm to room temperature and stirred overnight. The progress of reaction was monitored using TLC on silica gel. The reaction was quenched by pouring the mixture in water followed by extraction with diethyl ether (3 × 100 mL). The organic layers were combined and dried with sodium sulfate. The volatiles were evaporated under reduced pressure and the remaining liquid residue was purified by column chromatography on silica gel using hexanes–ethyl acetate (0-50 % ethyl acetate in hexanes) as eluent to afford the desired product as pale yellow liquid (1.24 g, 20 %); R_f = 0.41 (hexanes : ethyl acetate 7:3 *v/v*); IR (neat, cm^{-1}) 3382 (O-H H-bonded), 3288 (alkyne C-H stretch), 2953 (aromatic C-H stretch), 2872, 2128 (alkyne C-C stretch), 1709, 1462, 1364, 1102, 669;^{1}H NMR (500 MHz, $CDCl_3$) δ 4.12 (d, *J* = 2.74 Hz, 2H, CH_2OCH_2CCH), 3.60 (t, *J* = 6.62 Hz, 2H, -OCH_2CH_2-), 3.49 (t, *J* = 6.62 Hz, 2H), 2.42 (t, *J* = 2.28 Hz, 1H, -CH_2OCH_2CCH), 1.96 (br, s, 1H), 1.60-1.51 (m, 4H), 1.36-1.30 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 80.0, 74.0, 70.2, 62.8, 57.9, 32.6, 29.4,29.3, 29.2, 25.9, 25.6.

4-(8-(prop-2-ynyloxy)octyloxy)benzaldehyde (6b). To a solution of *p*-hydroxybenzaldehyde (0.82 g, 6.73 mmol) in dry dichloromethane-dioxane (30.0 mL, dichloromethane: dioxane 2:1,

v/v), triphenyl phosphine (2.64 g, 10.1 mmol) and 8-(prop-2-ynyloxy) octan-1-ol (**6a**) (1.24 g, 6.73 mmol) were added and the solution was ice cooled. To this solution, diisopropylazodicarboxylate (DIAD) (2.03 g, 10.1 mmol) was added at 0 ^{o}C slowly over 15 min. The contents were initially stirred at 0 ^{o}C for 30 min and then at room temperature overnight. Progress of the reaction was monitored by thin layer chromatography on silica gel. The solvents were removed under reduced pressure. The gummy residue was dissolved in 40.0 mL ethyl acetate:hexane (1:1 *v/v*) and left in the refrigerator for a day. The precipitated solid was filtered off and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes-ethyl acetate as eluent to afford the desired compound as white solid (1.18 g, 40 %): R_f = 0.67 (hexanes:ethyl acetate 7:3 *v/v)*; mp 50-52 ºC; IR (neat, cm^{-1}) 3293 (alkyne C-H stretch), 2993 (aromatic C-H stretch), 2855, 1695 (C=O stretch), 1601, 1580, 1511, 1319, 1249, 1155, 1094, 1008, 829, 641; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.91 (s, 1H), 7.85 (d, *J* = 8.60 Hz, 2H), 7.01 (d, *J* = 8.60 Hz, 2H), 4.16 (d, *J* = 2.20 Hz, 2H, -CH_2OCH_2CCH), 4.06 (t, *J* = 5.96 Hz, 2H, -OCH_2CH_2-), 3.54 (t, *J* = 6.39 Hz, 2H), 2.44 (t, *J*= 1.99 Hz, 1H, -CH_2OCH_2CCH), 1.86-1.81 (m, 2H), 1.64-1.60 (m, 2H), 1.53-1.47 (m, 2H), 1.43-1.36 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.7, 164.2, 131.9, 129.8, 114.7, 80.0, 74.1, 70.2, 68.4, 58.0, 29.4, 29.3, 29.2, 29.0, 26.0, 25.8.

N-methoxy-N-methyl-2-(4-(8-(prop-2-ynyloxy)octyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (6c). To solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.88 g, 3.47 mmol) in ethanol-ethyl acetate mixture (40.0 mL, 3:1 *v/v*), Pd-C (10%) (0.30 g) was added. Hydrogenation for 5h at the atmospheric pressure yielded corresponding diamine which was, after filtration of catalyst, used without further characterization (R_f = 0.46 in dichloromethane:methanol 9:1, *v/v*). 4-(8-(prop-2-ynyloxy) octyloxy) benzaldehyde (**6b**) (1.00 g,

3.47 mmol) and sodium metabisulfite (0.66 g, 3.47 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 6 h. Volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol as eluent to afford the desired product as sticky pale yellow sticky solid (1.35 g,87 %): R_f = 0.75 (in dichloromethane-methanol 9:1 *v/v*); IR (neat, cm^{-1}) 3432 (N-H wagging), 2946 (aromatic C-H stretch), 2853, 2112 (alkyne C-H stretch), 1630 (C=O stretch), 1256, 1175, 1101; ^{1}H NMR (500 MHz, acetone-d_6) δ 12.27 (br, 1H), 8.19 (dd, J_1= 8.83 Hz, J_2 = 2.05 Hz, 2H), 7.97 (br, 1H), 7.60 (2H), 7.06 (d, J_1 = 8.77, J_2 = 2.53, 2H), 4.13 (d, J = 2.14 Hz, 2H, -CH_2OCH_2CCH), 4.05 (t, J = 6.43 Hz, 2H, -OCH_2CH_2), 3.61 (s, 3H), 3.49 (t, J = 6.43 Hz, 2H), 3.35 (s, 3H), 2.94 (t, J = 2.14 Hz, 1H, -CH_2OCH_2CCH), 1.82-1.78 (m, 2H), 1.60-1.55 (m, 2H), 1.51-1.45 (m, 2H), 1.41-1.32 (m, 6H) ; ^{13}C NMR (125 MHz, acetone-d_6) 170.1, 161.0, 153.4, 135.7, 128.3, 128.2, 122.7, 122.5, 114.8, 80.3, 74.5, 71.1, 69.9, 69.5, 67.9, 60.2, 57.4, 33.3, 29.3, 29.2, 29.0; MS (MALDI-TOF) *m/z* calcd. for $C_{27}H_{33}N_3O_4$ $[M]^+$ 463.24, found 464.57.

2-(4-(8-(prop-2-ynyloxy)octyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (6d). To a solution of *N*-methoxy-*N*-methyl-2-(4-(8-(prop-2-ynyloxy)octyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**6c**) (1.21 g, 2.61 mmol) in THF-ether (80.0 mL, 3:1), lithium aluminum hydride (0.29 g, 7.83 mmol) was added at -78 ^{o}C under argon and then allowed to stir at 0 ^{o}C for 8h . The reaction mixture was quenched by the addition of saturated ammonium chloride solution (100 mL). The resulting grey precipitate was filtered off. The filtrate was extracted with ethyl acetate (3 × 50 mL). Organic layers were combined and then dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using ethyl acetate-hexane (0-80 % ethyl acetate in hexanes) as eluent to give the desired compound as light yellow liquid (0.85 g, 81 %): R_f = 0.73 (ethyl

acetate-hexane 8:2 *v/v*);mp 84-86 ºC;IR (neat, cm^{-1}) 3411 (N-H wagging), 3304 (alkyne C-H stretch), 2928 (aromatic C-H stretch), 2839, 2107 (alkyne C-C stretch), 1719 (C=O stretch), 1637, 1469, 1339, 1106, 938, 717, 628; ^{1}H NMR (500 MHz, methanol-d_4) δ 9.94 (s, 1H), 8.02 (s, br, 1H), 7.93 (dd, J_1 = 8.76 Hz, J_2 = 1.89 Hz), 2H), 7.74 (dd, J_1 = 8.08 Hz, J_2 = 1.25 Hz, 1H), 7.62 (s, br, 1H), 6.95 (dd, J_1 = 8.84 Hz, J_2 = 1.82 Hz, 2H) , 4.11 (d, J = 2.38 Hz, 2H, -CH_2OCH_2CCH), 3.88 (t, J = 6.57 Hz, 2H, -OCH_2CH_2-), 3.48 (t, J = 6.34 Hz, 2H) 2.83 (t, J = 2.38 Hz, 1H, -CH_2OCH_2CCH), 1.72-1.67 (m, 2H), 1.56-1.51 (m, 2H), 1.41-1.26 (m, 8H); ^{13}C NMR (75 MHz, methanol-d_4) δ 192.3, 161.4, 131.6, 128.2, 123.7, 120.8, 114.6, 79.5, 74.2, 69.6, 67.8, 57.3, 29.13, 29. 07, 29.05, 28.9, 25.7, 25.6; MS (MALDI-TOF) *m/z* for calcd for $C_{25}H_{28}N_2O_3$ 404.21, found 406.56 $[M+2H]^+$.

6-(4-methylpiperazin-1-yl)-2'-(4-(8-(prop-2-ynyloxy) octyloxy) phenyl)-1H, 3'H-2, 5'-bibenzo[d]imidazole (6). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.35 g, 1.50 mmol) in ethanol- ethyl acetate mixture (40.0 mL, 3:1 *v/v*), Pd-C (0.10 g) was added and then it was hydrogenated for 5 h at the atmospheric pressure. Catalyst was filtered off. To this solution, 2-(4-(8-(prop-2-ynyloxy) octyloxy) phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**6d**) (0.61 g, 1.50 mmol) and a solution of $Na_2S_2O_5$ (0.28 g, 1.50 mmol) in water (1.00 mL) was added. The mixture was refluxed overnight. The reaction mixture was brought to room temperature. Volatiles were removed reduced pressure. The crude mixture was purified by column chromatography on a silica gel column using dichloromethane-methanol (0-15 % methanol in dichloromethane) as eluent to afford the desired product as yellow solid (0.62 g, 71%): R_f = 0.35 (dichlormethane-methanol 8:2 *v/v*);mp 174-176 ºC;IR (neat, cm^{-1}) 3405 (N-H wagging), 2927 (aromatic C-H stretch), 2846, 1634 (C=O stretch), 1437, 1256, 1178, 1086;^{1}H NMR (500 MHz, methanol-d_4) δ 8.18 (br, 1H) 7.91-7.89 (m, 3H), 7.63 (d, J = 7.65 Hz, 1H),

7.48 (d, J = 8.20 Hz, 1H), 7.09 (s, br, 1H), 7.00 (dd, J_1 = 8.47 Hz, J_2 = 1.91 Hz, 1H), 6.89 (d, J = 8.46 Hz, 2H), 4.11 (d, J = 2.19 Hz , 2H, -CH_2OCH_2CCH), 3.80 (t, J = 6.48 Hz, 2H, -OCH_2CH_2-), 3.47 (t, J = 6.28 Hz, 2H), 3.19 (t, J = 3.55 Hz, 4H), 2.84 (t, J = 2.45 Hz, 1H, -CH_2OCH_2CCH), 2.65 (t, J = 4.10 Hz, 4H), 2.37 (s, 3H), 1.66-1.61 (m, 2H), 1.55-1.50 (m, 2H), 1.35-1.22 (m, 8H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.1, 153.8, 152.3, 146.1, 134.8, 133.1, 120.8, 124.1, 121.2, 120.9, 115.6, 114.8, 114.4, 100.5, 79.5, 74.1, 71.4, 70.3, 69.6, 67.7, 67.4, 57.3, 54.7, 50.3, 48.4, 44.6, 29.1, 29.06, 29.03, 28.8, 25.7, 25.6; ESI-HRMS *m/z* calcd for $C_{36}H_{43}N_6O_2$ 591.3444, found 591.3448 $[M]^+$; HPLC: t_R 5.93 min, purity 97.8%.

10-(prop-2-ynyloxy) decan-1-ol (7a).To a solution of 1, 10 decanediol (4.00 g, 22.9 mmol) in dry THF, sodium hydride (0.48 g, 20.0 mmol) was added under argon at 0 ^{o}C. The mixture was stirred for 30 min at 0 ^{o}C. To this, propargyl bromide (2.37 g, 20.0 mmol) was added and stirred at 0 ^{o}C for another 30 min and then at room temperature for 24h. The progress of reaction was monitored using TLC on silica gel. The reaction mixture was quenched by pouring the mixture into water. The mixture was extracted with diethyl ether (3 × 100 mL). The organic layers were combined at dried with sodium sulfate. The volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes-ethyl acetate (0-50 % ethyl acetate in hexanes) as eluent to afford the desired product (2.40 g, 50 %): R_f = 0.74 (hexanes-ethyl acetate 60:40 *v/v*); IR (neat, cm^{-1}) 3411 (O-H H-bonded), 3304 (alkyne C-H stretch), 2928 (aromatic C-H stretch), 2107 (alkyne C-C stretch), 1719, 1637, 1469, 1339, 1106, 938, 717, 628; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.14 (d, J = 2.4 Hz, 2H, -CH_2OCH_2CCH), 3.63 (t, J = 6.2 Hz, 2H, -OCH_2CH_2-), 3.51 (t, J = 6.5 Hz, 2H), 2.43 (t, J = 2.3 Hz, 1H, -

CH_2OCH_2CCH), 1.64-1.52 (m, 4H), 1.40-1.30 (br, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 80.0, 76.4, 74.0, 70.2, 62.9, 57.9, 32.7, 29.5, 29.4, 29.3, 26.0, 25.7.

4-(10-(prop-2-ynyloxy)decyloxy)benzaldehyde (7b). To a solution of *p*-hydroxybenzaldehyde (1.00 g, 8.18 mmol) in dry dichloromethane-dioxane mixture (40.0 mL 3:1 *v/v*), triphenyl phosphine (3.20 g, 12.1 mmol) and 10-(prop-2-ynyloxy)decan-1-ol (**7a**) (1.73 g, 8.18 mmol) were added and the solution was ice cooled. To this, diisopropylazodicarboxylate (2.44 g, 12.1 mmol) was added at 0 ^oC slowly over 15 min. The contents were initially stirred at 0 ^oC for 30 minutes and then at room temperature overnight. Progress of the reaction was monitored by thin layer chromatography on silica gel. The crude mixture was concentrated and re-dissolved in ethyl acetate-hexanes mixture {80.0 mL, 1:1 (*v/v*)} and kept in the refrigerator for a day. The solid that crashed out was filtered off and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (hexanes-ethyl acetate (0-50 % ethyl acetate in hexanes) afforded the desired compound as white solid (0.93 g, 36 %): R_f = 0.73 (hexanes-ethyl acetate 7:3);mp 52-53 ºC; IR (neat) cm^{-1} 3251 (alkyne C-H stretch), 2942 (aromatic C-H stretch), 2853, 2116 (alkyne C-C stretch), 1692 (C=O stretch), 1599, 1499, 1252, 1159, 1093, 1001. 835; ^1H NMR (300 MHz, $CDCl_3$) δ 9.88 (s, 1H), 7.83 (dd, J_1 = 8.8 Hz, J_2 = 1.9 Hz, 2H), 6.99 (d, J = 8.7 Hz, 2H), 4.14 (d, J = 2.4 Hz, 2H, -CH_2OCH_2CCH), 4.04 (t, J = 6.5 Hz, 2H, -OCH_2CH_2-), 3.51 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 2.30 Hz, 1H, -CH_2OCH_2CCH), 1.86-1.77 (m, 2H), 1.65-1.56 (m, 2H), 1.46-1.32 (br, 12H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.7, 164.2, 131.9, 129.7, 114.7, 80.0, 76.6, 74.0, 70.2, 68.4, 58.0, 30.9, 29.5, 29.4, 29.3, 29.2, 29.0, 26.2, 26.0, 25.9.

N-methoxy-N-methyl-2-(4-(10-(prop-2-ynyloxy)decyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (7c). To solution of *N*- Methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.64 g, 2.84 mmol) in ethanol-ethyl acetate mixture (20.0 mL 3:1 *v/v*), 0.20 g of 10 % Pd-C was added. It was then hydrogenated for 5h at the atmospheric pressure to afford corresponding diamine which was used without purification after filtration of the catalyst. 4-(10-(prop-2-ynyloxy)decyloxy)benzaldehyde (**7b**) (0.90 g, 2.84 mmol) and sodium pyrosulfite (0.54 g, 2.84 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed for 10 h. Volatiles were evaporated under educed pressure. The crude product was purified on a silica gel column using dichlromethane-methanol (0-8 % methanol in dichloromethane) as eluent to afford the desired product as slightly yellow solid (0.86 g, 70 %): R_f = 0.85 {dichloromethane-isopropanol 9:1 (*v/v*)}; IR (neat cm^{-1}) 2930 (aromatic C-H stretch), 2849, 2116 (alkyne C-C stretch), 1611, 1425, 1252, 1178, 1093, 1016, 819, 731; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.17 (d, J_1 = 8.8 Hz, J_2 = 1.9 Hz¸ 2H), 8.04-7.88(br,1H), 7.66- 7.53 (br, 3H), 7.09 (d, J_1 = 8.8 Hz, J_2= 1.9 Hz¸ 2H), 4.13 (d, *J* = 2.30 Hz, 2H, -CH_2OCH_2CCH), 4.08 (t, *J* = 6.5 Hz, 2H, -OCH_2CH_2-), 3.61 (s, 3H), 3.49 (t, *J* = 6.4 Hz¸ 2H), 3.34 (s, 3H), 2.92 (t, *J* = 2.4 Hz¸ 1H, -CH_2OCH_2CCH), 1.85-1.76 (m, 2H), 1.59-1.30 (m, 14H); ^{13}C NMR (75 MHz, acetone-d_6) δ 170.0, 160.9, 153.6, 153.1, 146.2, 136.7, 134.3, 128.2, 122.9, 122.5, 119.3, 117.9, 114.7, 111.5, 110.2, 80.3, 74.5, 69.5, 67.9, 60.2, 57.4, 33.2, 25.8; MS (MALDI-TOF) *m/z* calcd for C_{29} $H_{37}N_3O_4$ 491.27 found 492.35 $[M+H]^+$.

2-(4-(10-(prop-2-ynyloxy)decyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (7d). To a solution of *N*-methoxy, *N*-methyl-2-(4-(10-(prop-2-ynyloxy)decyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**7c**) (0.80 g, 1.62 mmol) in THF-ether (80.0 mL, 3:1 *v/v*), lithium aluminum hydride (0.18 g, 4.8 mmol) was added at -78 ^{o}C under argon and then allowed

to stir at 0 ^{o}C overnight . The reaction mixture was quenched by the addition of saturated ammonium chloride solution (75.0 mL). The resulting grey precipitate was filtered off. The filtrate was extracted with ethyl acetate (3 × 75 mL). Organic layers were combined and then dried over sodium sulfate. Volatiles were removed under reduced pressure. Column Chromatography on silica gel using hexanes-ethyl acetate (0- 80 % ethyl acetate in hexanes) as eluent yielded the desired compound as a light yellow solid (0.43 g, 61 %): R_f = 0.68 {ethyl acetate-hexanes 7:3 (v/v)}; mp 124-126 ºC; IR (neat, cm^{-1}) 3228 (alkyne C-H stretch), 2923 (aromatic C-H stretch), 2846, 1688 (C=O stretch), 1618, 1456, 1290, 1244, 1070, 850; ^{1}H NMR (300 MHz, acetone-d_6) δ 10.09 (s, 1H), 8.22-8.05 (br, 3H),7.82-7.62 (2H), 7.13 (d, J = 8.0 Hz, 2H), 4.12 (br, 4H), 3.49 (t, J = 6.4 Hz, 2H), 1.88-1.76 (m, 2H), 1.57-1.25 (m, 14H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 192.9, 161.1, 154.9, 131.4, 128.9, 123.4, 122.1, 115.3, 81.0, 77.2, 69.5, 68.2, 60.2, 57.7, 29.4, 29.3, 29.2 (two peaks), 26.0, 25.9; MS (MALDI-TOF) *m/z* calcd for $C_{27}H_{32}N_2O_3$ 432.24, found 434.23 ($[M+2H]^+$).

6-(4-methylpiperazin-1-yl)-2'-(4-(10-(prop-2-ynyloxy)decyloxy)phenyl)-1H,3'H-2,5'-bibenzo[d]imidazole (7). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.16 g, 0.78 mmol) in ethanol- ethyl acetate mixture (20.0 mL), 10 % Pd-C (80 mg) was added followed by hydrogenation for 5h at the atmospheric pressure. TLC on silica gel showed complete reduction of the starting material. Charcoal was filtered over a bed of celite. To the filtrate, 2-(4-(10-(prop-2-ynyloxy)decyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**7d**) (0.34 g, 0.78 mmol) and a solution of $Na_2S_2O_5$ (0.15 g, 0.78 mmol)in water (1.00 mL) was added and the mixture was refluxed for 14 h. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol (0-15 % methanol in dichloromethane) as eluent to afford the pure product as yellow solid (0.24

g, 55 %): R_f = 0.38 (dichloromethane - methanol 8:2 *v/v*); mp 162-164 ºC; IR (neat, cm^{-1}) 3397 (N-H wagging), 2930 (aromatic C-H stretch), 2846, 2112 (alkyne C-C stretch), 1630 (C=O stretch), 1452, 1252, 1182, 1097, 1009; ^{1}H NMR (300 MHz, methanol-d_4) δ 8.25 (br, 1H) 8.03-7.94 (br, 3H), 7.68 (d, br, 1H), 7.52 (d, br, 1H), 7.14 (br, 1H), 7.06-7.03 (m, 3H), 4.12 (t, *J* = 4.6Hz , 2H), 3.99 (br, 2H), 3.52-3.48 (2H), 3.24 (br, 4H), 2.83-2.81 (t, *J* = 2.18 Hz, 1H), 2.73 (br, 4H), 2.42 (s, 3H), 1.77-1.68 (m, 2H), 1.57-1.33 (14H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 160.7, 153.4, 147.9, 145.5, 144.6, 136.0, 128.7, 125.0, 122.7, 121.5, 120.8, 119.0, 116.4, 115.3, 111.8, 109.3, 81.0, 77.2, 69.6, 68.2, 61.6, 57.7, 55.2, 50.2, 49.0, 45.9, 29.6, 29.4, 29.2(2), 29.1, 26.0, 25.9, 15.6 ; ESI-HRMS *m/z* calcd. for $C_{38}H_{47}N_6O_2$619.3761, found 619.3761 $[M]^+$; HPLC: t_R 12.1 min, purity 95.1%.

12-(prop-2-ynyloxy) dodecan-1-ol *(*8a). To a solution of 1, 12 dodecanediol (6.00 g, 29.6 mmol) in dry THF, sodium hydride (0.64 g, 27.0 mmol) was added under argon at 0 ^{o}C. The mixture was stirred for 30 min at 0 ^{o}C. To this, propargyl bromide (3.21 g, 27.0 mmol) was added and stirred at 0 ^{o}C for another 30 min and then at room temperature for 24 h. The reaction was quenched by pouring it into water. It was then extracted with diethyl ether (3 × 100 mL). The organic layers were combined and dried with sodium sulfate. The volatiles were evaporated under reduced pressure. The crude mixture was purified on a silica gel column using petroleum ether-ethyl acetate as eluent to afford the desired product as brown oil (3.20 g, 49 %): R_f = 0.55 (hexanes-ethyl acetate 60:40 *v/v*); IR (neat, cm^{-1}) 3411 (O-H H-bonded), 3308 (alkyne C-H stretch), 2924 (aromatic C-H stretch), 2851, 2111 (alkyne C-C stretch), 1698, 1469, 1355, 1224, 1102, 664; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.07 (d, *J* = 2.3 Hz, 2H), 3.54 (t, *J* = 6.5 Hz, 2H), 3.45 (t, *J* = 6.5 Hz, 2H), 2.39 (t, *J* = 2.4 Hz, 1H), 1.58-1.44 (m, 4H), 1.24-1.17(br, 16H); ^{13}C NMR (75

MHz, $CDCl_3$) δ 79.9, 76.7, 74.1, 70.1, 62.6, 57.8, 32.6, 30.8, 29.55, 29.51, 29.4, 29.3, 29.1, 26.0, 25.7.

4-(12-(prop-2-ynyloxy)dodecyloxy)benzaldehyde (8b). To a solution of *p*-hydroxybenzaldehyde (1.00 g, 8.18 mmol) in dry dichloromethane-1,4dioxane (40.0 mL, 3:1 *v/v*), triphenyl phosphine (3.17 g, 12.1 mmol) were added under argon and the solution was ice cooled. 12-(prop-2-ynyloxy)dodecan-1-ol (**8a**) (1.96 g, 8.18 mmol) was added into it. To this, diisopropylazodicarboxylate (DIAD) (2.44 g, 12.1 mmol) was added at 0 ^oC slowly over 15 min. The contents were initially stirred at 0 ^oC for 30 min and then at room temperature overnight. Volatiles were removed under reduced pressure. The mixture was re-dissolved in ethyl acetate-hexanes (80.0 mL, 1:1 *v/v*). The crude mixture was then allowed to stand in the refrigerator for a day. The solid crashed out was filtered off and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using petroleum ether-ethyl acetate (0-80 % ethyl acetate in petroleum ether) as eluent to afford the desired compound as white solid (0.82 g, 30 %): R_f = 0.75 (petroleum ether-ethyl acetate 8:2 *v/v*); ^1H NMR (300 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.83 (dd, J_1 = 8.8 Hz, J_2 = 2.5 Hz, 2H,), 7.00 (d, J = 8.7 Hz, 2H), 4.15 (d, J = 2.4 Hz, 2H), 4.05 (t, J = 6.5 Hz, 2H), 3.52 (t, J = 6.6 Hz, 2H), 2.43 (t, J = 2.30 Hz, 1H), 1.86-1.78 (m, 2H), 1.65-1.56 (m, 2H), 1.46-1.20 (br, 16H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.8, 164.2, 131.9, 129.7, 114.7, 80.0, 74.0, 70.3, 68.4, 58.0, 29.5 (five peaks), 29.4, 29.3, 29.0, 26.0, 25.9.

N-methoxy-N-methyl-2-(4-(12-(prop-2-ynyloxy)dodecyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (8c). To solution of *N*-methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.59 g, 2.31 mmol) in ethanol-ethyl acetate mixture (20.0 mL, 1:1 *v/v*), 10% Pd-C (0.20 g) was added. Hydrogenation for 5 h at the atmospheric pressure afforded

corresponding diamine which was (after filtration of the catalyst) used without purification. 4-(12-(prop-2-ynyloxy)dodecyloxy)benzaldehyde (**8b**) (0.97 g, 2.31 mmol) and sodium metabisulfite (0.43 g, 2.31 mmol) in water (1.00 mL) were added into it and the reaction mixture was refluxed overnight. Volatiles were evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-isopropanol (0-10 % isopropanol in dichloromethane) as eluent to afford the desired product as sticky yellow solid (0.32 g, 27 %): R_f = 0.64 (dichloromethane-isopropanol 9:1 *v/v*); ^{1}H NMR (500 MHz, acetone-d_6) δ 12.0 (s, br, 1H), 8.18 (d, *J* = 8.8 Hz, 2H), 8.05-7.87(br, 1H), 7.67- 7.53 (br, 2H), 7.10 (d, J_1= 8.8 Hz, J_2= 2.0 Hz, 2H), 4.13-4.08 (m, 4H), 3.62 (s, 3H), 3.49 (t, *J* = 6.6 Hz, 2H), 3.35 (s, 3H), 2.92 (t, *J* = 2.4 Hz, 1H), 1.85-1.79 (m, 2H), 1.59-1.48 (m, 4H), 1.42-1.28 (14H); ^{13}C NMR (125 MHz, acetone-d_6) δ 170.0, 160.9, 131.8, 128.7, 128.6, 128.2, 122.9, 122.5, 114.8 (two peaks), 111.5, 110.1, 80.3, 74.5, 69.5, 67.9, 60.1, 57.4, 33.2, 28.5, 21.; MS (MALDI-TOF) *m/z* calcd for $C_{31}H_{41}N_3O_4$ 519.30 found 520.40 $[M+H]^+$.

2-(4-(12-(prop-2-ynyloxy)dodecyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (8d). To a solution of *N*-methoxy-*N*-methyl-2-(4-(12-(prop-2-ynyloxy)dodecyloxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (**8c**) (0.30 g, 0.57 mmol) in THF-ether (20.0 mL, 2:1 *v/v*), lithium aluminum hydride (0.06 g, 1.70 mmol) was added at -78 ^{o}C under argon and then allowed to stir at 0 ^{o}C for 24 h . TLC on silica gel was used to monitor the progress of the reaction. The reaction mixture was quenched by addition of saturated ammonium chloride solution (50.0 mL). The resulting grey precipitate was filtered off. The aqueous mixture was extracted with ethyl acetate (3 × 75 mL). Organic layers were combined and then dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using hexanes:ethyl acetate (0-80 % ethyl acetate in hexanes) as

eluent to afford the desired compound as a light yellow solid (0.24 g, 91 %): R_f = 0.63 (hexanes-ethyl acetate 4:6 *v/v*); ^{1}H NMR (500 MHz, acetone-d_6) δ 10.08 (s, 1H), 8.22-8.06 (br, 3H),7.81-7.79 (2H), 7.11 (d, *J* = 8.9 Hz, 2H), 4.13-4.08 (m, 4H), 3.48 (t, *J* = 6.5 Hz, 2H) 2.92 (t, *J* = 2.4 Hz, 1H), 1.86-1.77 (m, 2H), 1.58-1.45 (m, 2H), 1.41-1.26 (m, 16H); ^{13}C NMR (125 MHz, acetone-d_6) δ 191.4, 161.3, 144.5, 132.5, 131.8, 131.1, 128.7, 128.4, 123.0, 122.0, 119.0, 114.8, 113.3, 80.2, 74.5, 69.5, 67.9, 67.4, 57.3, 38.7, 30.2, 26.0, 25.8, 23.6, 22.7, 21.3, 13.4; MS (MALDI-TOF) *m/z* calcd for $C_{29}H_{36}N_2O_3$ 460.27 found 461.47.

6-(4-methylpiperazin-1-yl)-2'-(4-(12-(prop-2-ynyloxy)dodecyloxy)phenyl)-1H,3'H-2,5'-bibenzo[d]imidazole (8). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.12 g, 0.50 mmol) in ethanol- ethyl acetate mixture (20 mL 1:1 *v/v*), Pd-C (75 mg) was added and then it was hydrogenated for 5 h at the atmospheric pressure. TLC showed complete reduction of the starting material to its corresponding diamine. After filtering the catalyst, 2-(4-(12-(prop-2-ynyloxy)dodecyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**8d**) (0.24 g, 0.52 mmol) and a solution of $Na_2S_2O_5$ (0.09 g, 0.50 mmol)in water (0.50 mL) were added. The mixture was refluxed for 20h. It was then allowed to come to the room temperature. Volatiles were removed under reduced pressure. The crude mixture was purified on a silica gel column using dichloromethane-methanol (0-15 % methanol in dichloromethane) to afford the desired product as yellow solid (0.13 g, 40 %): R_f = 0.37 (dichloromethane-methanol 8:2 *v/v*); mp 135-140 ºC; IR (neat, cm^{-1}) 3382 (alkyne C-H stretch), 2969 (aromatic C-H stretch), 1649, 1576, 1468, 1240, 1005; ^{1}H NMR (300 MHz, methanol-d_4) δ 8.23 (br, 1H) 8.02-7.99 (br, 2H), 7.92 (d, *J* = 7.5 Hz, 1H), 7.68-7.65 (d, *J* = 7.9 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.13-7.02 (4H), 4.09-3.98 (4H), 3.48 (2H), 3.20 (t, *J* = 4.3Hz, 4H), 2.90-2.79 (5H) 2.14 (s, 3H), 1.84-1.66 (m, 2H),1.60-1.20(m, 18H) (Imino protons were not observed likely because of the exchange with the NMR solvent); ^{13}C

NMR (125 MHz, methanol-d_4) δ 161.8, 148.0, 128.1, 124.2, 121.4, 115.0, 114.6, 79.4, 74.0, 69.6, 67.8, 57.2, 54.6, 50.1, 29.2, 25.6 (all carbon atoms were not observed because of aggregation effects); ESI-HRMS*m/z*calcd for $C_{40}H_{51}N_6O_2$ 647.4074 found 647.4072 $[M+H]^+$;HPLC: t_R 12.9 min, purity 99.0%.

3,6,9,12,15,18-hexaoxahenicos-20-yn-1-ol (9a).To a solution of hexaethylene glycol in dry THF (10.0 g, 35.4 mmol) at 0 ^oC, sodium hydride (0.77 g, 32 mmol) was added and the mixture was stirred for 30 min. To this, propargyl bromide (3.8 g, 32 mmol) was added and the mixture was allowed to warm up to room temperature. The stirring was continued for 20 h. Water (200 mL) was added to this mixture and then extracted with ethyl acetate (3 × 200 mL). Organic layers were combined and dried with sodium sulfate. Volatiles were evaporated under reduced pressure. and the crude product was purified using ethyl acetate-acetone as eluent to afford the desired compound as light brown oil (4.8 g, 46 %): R_f = 0.39 (ethyl acetate: acetone 7:3 *v/v*); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.16 (d, *J* = 2.00 Hz, 2H), 3.69-3.55 (m, 24H), 2.85 (t, *J* = 5.75 Hz, 1H), 2.43 (t, *J* = 2.25 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 79.6, 74.5, 72.5, 70.5, 70.3, 70.3, 70.2, 69.0, 61.6, 58.3.

4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)benzaldehyde (9b). To a solution of *p*-hydroxybenzaldehyde (1.50 g, 12.5 mmol) in dry dichloromethane-dioxane (50.0 mL, 4:1 *v/v*), triphenyl phosphine (4.70 g, 18.0 mmol) and 3,6,9,12,15,18-hexaoxahenicos-20-yn-1-ol (**9a**) (4.00 g, 12.5 mmol) were dissolved and the solution was ice cooled. To this, diisopropylazodicarboxylate (3.60 g, 18.0 mmol) was added drop wise over a period of 15 min at 0 ^oC. The contents were initially stirred at 0 ^oC for 30 min and then at room temperature overnight. The volatiles were removed under reduced pressure and the mixture was re-dissolved in ethyl acetate-hexane (60.0 mL, 1:1 *v/v*) and allowed to sit in the refrigerator overnight. The

precipitated solid was filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using hexanes-ethyl acetate (0-100% ethyl acetate in hexane) and then ethyl acetate-acetone (0-10% *v/v* acetone in ethyl acetate) as eluent, afforded the desired compound as pale yellow oil (4.80 g, 90 %): R_f = 0.63 (ethyl acetate-acetone 7:3 *v/v)*;IR (neat cm^{-1}) 3247 (alkyne C-H stretch) , 2871 (aromatic C-H stretch), 2111 (alkyne C-C stretch), 1694 (C=O stretch), 1600, 1502, 1437, 1347, 1261, 1110, 832, 726, 542; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.85(s, 1H), 7.80 (dd, J_1 = 1.82 Hz, J_2 = 8.80 Hz, 2H), 7.00 (dd, J_1 = 1.73 Hz, J_2 = 8.62 Hz, 2H), 4.18 (m, 4H), 3.86 (t, J = 4.88 Hz, 2H), 3.72-3.62 (m, 20H), 2.44 (t, J = 2.59 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.7, 163.8, 131.9, 130.0, 114.8, 79.6, 74.5, 70.8, 70.6, 70.5, 70.3, 69.4, 69.0, 67.7, 58.3.

2-(4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)phenyl)-N-methoxy-N-methyl-1H-benzo[d]imidazole-6-carboxamide (9c). To a solution of *N*- methoxy, *N*-methyl 3, 4 dinitrobenzamide (0.90 g, 3.53 mmol) in ethanol-ethyl acetate mixture (50.0 mL, 3:1 *v/v*), Pd-C (10%) (400 mg) was added. Hydrogenation for 5 h at the atmospheric pressure yielded corresponding diaminewhich was used without further characterization after filtration of the catalyst over a bed of celite (R_f = 0.46 in dichloromethane:methanol 9:1,*v/v*). 4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)benzaldehyde (**9b**) (1.5 g, 3.53 mmol) and sodium metabisulfite (0.67 g, 3.53 mmol) in water (1.00 mL) was added into it and the reaction mixture was refluxed for 6 h. The reaction mixture was allowed to come to room temperature. The volatiles were evaporated under reduced pressure. The crude product was purified on a silica gel column using dichloromethane-methanol (0-10% methanol in dichloromethane) as eluent to afford the desired product as pale yellow solid (0.85 g,41 %): R_f = 0.48 (in dichloromethane:me thanol 9:1 *v/v*); IR (neat, cm^{-1}) 3247 (alkyne C-H stretch), 2876 (aromatic C-H stretch), 2116 (alkyne C-C stretch),

1614, 1449, 1259, 1101, 843; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.06 (dd, J_1= 8.79 Hz, J_2 = 2.04 Hz, 2H), 7.96 (br, 1H), 7.64 (d, J = 8.18 Hz, 1H), 7.59 (dd, J_1 = 8.38 Hz, J_2 = 1.53 Hz, 1H), 7.12 (dd, J_1 = 8.79, J_2 = 1.84, 2H), 4.21 (t, J = 4.30 Hz, 2H), 4.17 (d, J = 2.35 Hz, 2H), 3.87 (m, 2H), 3.72-3.60 (m, 23H), 3.42 (s, 3H), 2.86 (t, J = 2.25 Hz, 1H); ^{13}C NMR (75 MHz, methanol-d_4) δ 170.5, 161.0, 154.0, 134.6, 131.3, 128.2, 127.7, 122.7, 121.6, 115.8, 114.8, 113.7, 79.2, 74.5, 70.40, 70.21, 70.15, 70.12, 69.93, 69.32, 69.15, 68.69, 67.46, 60.1, 57.6, 33.3 (some carbon peaks are overlapped on each other); MS (MALDI-TOF) *m/z* calcd. for $C_{31}H_{41}N_3O_9$ $[M]^+$ 599.18, found 599.27 ($[M]^+$).

2-(4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (9d). To a solution of 2-(4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)phenyl)-*N*-methoxy-*N*-methyl-1H-benzo[d]imidazole-6-carboxamide (**9c**) (0.85 g, 1.41 mmol) in THF-ether (100 mL, 3:1 *v/v*), lithium aluminum hydride (0.32 g, 8.40 mmol) was added at -78 ^{o}C under argon and then allowed to stir at 0 ^{o}C overnight . The reaction mixture was quenched by addition of saturated ammonium chloride solution (50.0 mL). The resulting grey precipitate was filtered off. The filtrate was extracted with ethyl acetate (3 × 100 mL). Organic layers were combined and then dried over sodium sulfate. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using dichloromethane-isopropanol as eluent to give the desired compound yielded the as a light yellow liquid (0.42 g, 56 %): R_f = 0.72 (dichloromethane-isopropanol 9:1 *v/v*); IR (neat, cm^{-1}) 2871 (aromatic C-H stretch), 1972, 1690 (C=O stretch), 1604, 1498, 1441, 1290, 1249, 1106, 954, 852; ^{1}H NMR (500 MHz, methanol-d_4) δ 10.03 (s, 1H), 8.13 (s, br, 1H), 8.07 (dd, J_1 = 8.82 Hz, J_2 = 1.93 Hz, 2H), 7.84 (dd, J_1 = 8.32 Hz, J_2 = 1.33 Hz, 1H), 7.72 (s, br, 1H), 7.14 (dd, J_1 = 8.74 Hz, J_2 = 1.96 Hz, 2H) , 4.23 (t, J = 4.26 Hz, 2H), 4.18 (d, J = 2.19 Hz, 2H), 3.89 (m, 2H), 3.74-3.60 (m, 20H) 2.86-2.82

(t, J = 2.41 Hz, 1H); ^{13}C NMR (75 MHz, methanol-d_4) δ 192.4, 161.2, 155.3, 134.6, 131.7, 128.3 (two peaks), 123.7, 121.3, 115.8, 115.8, 114.8, 79.2, 74.5, 71.6, 70.40, 70.21, 70.15, 69.93, 69.30, 69.14, 68.6, 67.4, 57.6; MS (MALDI-TOF) *m/z* calcd for $C_{29}H_{36}N_2O_8$ 540.24, found 541.08 ($[M+H]^+$) .

2'-(4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzo[d]imidazole (9). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.18 g, 0.78 mmol) in ethanol- ethyl acetate mixture (40.0 mL, 3:1 *v/v*), Pd-C (0.10 g) was added and then it was hydrogenated for 5h at the atmospheric pressure. Catalyst was filtered off. To this solution, 2-(4-(3,6,9,12,15,18-hexaoxahenicos-20-ynyloxy)phenyl)-1H-benzo[d]imidazole-6-carbaldehyde (**9d**) (0.42 g, 0.78 mmol) and a solution of $Na_2S_2O_5$ (0.15 g, 0.78 mmol)in water (1.00 mL) was added and the mixture was refluxed overnight. The reaction mixture was brought to room temperature. Volatiles were removed under reduced pressure. The crude mixture was purified by column chromatography on a silica gel and eluted with dichloromethane-methanol (0-20 % methanol in dichloromethane) to afford the desired product as yellow solid (0.38 g, 66%): R_f = 0.44 (dichlormethane-methanol 8:2 *v/v*);mp 134-138 ºC; IR (neat, cm^{-1}) 3402 (N-H wagging), 3186 (alkyne C-H stretch), 2871 (aromatic C-H stretch), 1968, 1633, 1612, 1490, 1453, 1290, 1245, 1102, 844; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.23 (br, 1H) 8.00 (d, J = 8.82 Hz, 2H), 7.93 (dd, J_1 = 8.58 Hz, J_2 = 1.45 Hz, 1H), 7.66 (d, J = 8.38 Hz, 1H), 7.50 (d, J = 8.71 Hz, 1H), 7.12 (d, J = 1.78 Hz, 1H), 7.02 (m, 3H), 4.16 (d, J = 2.51 Hz, 2H), 4.11 (t, J = 4.16 Hz, 2H), 3.80 (t, J = 4.36 Hz, 2H), 3.69-3.52 (m, 20H), 3.22 (t, J = 4.09 Hz, 4H), 2.85 (t, J = 2.50 Hz, 1H), 2.71 (t, J = 4.56 Hz, 4H), 2.41 (s, 3H); ^{13}C NMR (75 MHz, methanol-d_4) δ 159.3, 152.2, 150.7, 146.5, 146.3, 137.5, 133.0, 126.6, 122.6, 120.1, 119.5, 114.3,

113.6, 113.5, 113.3, 113.1, 111.0, 99.3, 77.6, 73.0, 71.1, 70.0, 68.79, 68.59, 68.53, 68.48, 68.32, 67.73, 67.53, 67.08, 65.7, 62.0, 56.0, 53.1, 48.6, 42.9, 27.3 ; ESI-HRMS *m/z* cald for $C_{40}H_{51}N_6O_7$ 727.3819, found 727.3818 $[M]^+$;HPLC: t_R 2.43 min, purity 99.1%.

2-(4-ethoxyphenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole (12). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.50 g, 2.12 mmol) in ethanol-ethyl acetate (2:1 *v/v*) mixture (20.0 mL), Pd-C (150 mg) was added and the mixture was hydrogenated for 4 h at the atmospheric pressure, which afforded the corresponding diamine. Catalyst was filtered off. To this, 4-ethoxy benzaldehyde (0.32 g, 2.12 mmol) and an aqueous solution of $Na_2S_2O_5$ (0.40 g, 2.12 mmol) were added, and the mixture was refluxed overnight. The mixture was brought to room temperature and volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent to afford the desired product as off white solid (0.34 g, 47%): R_f = 0.55 (dichloromethane-methanol 8:2 v/v); mp 165–170 ^oC; IR (neat) cm^{-1} 3423 (N-H wagging), 2970 (aromatic C-H stretch), 1629, 1449, 1245, 1171, 1036; ^1H NMR (500 MHz, acetone-d_6) δ 9.89 (s, 1H), 8.13 (dd, J_1= 8.8 Hz, J_2= 2.8 Hz, 2H), 7.44 (d, J = 8.6 Hz, 1H), 7.04-7.02 (3H), 6.95 (dd, J_1= 2.3Hz, J_2= 8.8 Hz, 1H), 4.12 (q, 2H), 3.15 (t, J = 4.89 Hz, 4H), 2.57 (t, J = 5.0 Hz, 4H), 2.31 (s, 3H), 1.39 (t, J = 6.94 Hz, 3H); ^{13}C NMR (75 MHz, DMSO–d_6) δ 160.0, 151.0, 148.0, 130.8, 128.1, 123.3, 115.1, 114.0, 63.7, 55.2, 50.3, 46.0, 15.0; MS (MALDI-TOF) *m/z* calcd. for $C_{20}H_{24}N_2O$ 336.43, found 336.57 $[M]^+$; HPLC: t_R5.45 min, purity 97.5%.

4-(1H-benzo[d]imidazol-2-yl)phenol (13). To a solution of 5-chloro 2-nitroaniline (0.52 g, 3.01 mmol) in a mixture of ethanol–ethyl acetate (20.0 mL), Pd-C (0.20 g) was added. The mixture

was hydrogenated for 5 h at atmospheric pressure, which afforded the dehalogenated diamine product. Catalyst was filtered off. To this, 4-hydroxybenzaldehyde (0.37 g, 3.01 mmol) was added followed by addition of sodium metabisulfite (0.57 g, 3.01 mmol). The mixture was refluxed overnight. Volatiles were removed under reduced pressure. The crude product was purified on a silica gel column using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent to afford the desired compound as a beige solid (0.54 g, 84%): R_f = 0.80 (dichloromethane-methanol 8:2 v/v) ; mp>260 ^{o}C; IR (neat) cm^{-1} 3366, 3194, 1604, 1510, 1457, 1384, 1277, 1171, 844; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.9 (s, 1H), 8.32 (d, J = 8.2 Hz, 2H), 7.78 (q, 2H), 7.49 (q, 2H), 7.09 (d, J = 8.8 Hz, 2H); ^{13}C NMR (75 MHz, DMSO–d_6)) δ 162.5, 149.3, 133.0, 130.5, 125.4, 116.8, 114.6, 114.1; MS (MALDI-TOF) *m/z* calcd. For $C_{13}H_{10}N_2O$211.08, found 212.11 $[M+H]^+$;HPLC: t_R 4.64 min, purity 97.7%.

5-(4-methylpiperazin-1-yl)-2-(4-(6-(prop-2-ynyloxy)hexyloxy)phenyl)-1H-benzo[d]imidazole (14). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.11 g, 0.46 mmol) in ethanol-ethyl acetate (2:1 *v/v*) mixture (10.0 mL), 10% Pd-C (0.06 g) was added and the mixture was hydrogenated for 4 h at atmospheric pressure, which afforded the corresponding diamine. Catalyst was filtered off. To this, 4-(6-(prop-2-ynyloxy)hexyloxy) benzaldehyde (0.12 g, 0.46 mmol) and an aqueous solution of $Na_2S_2O_5$ (0.09 g, 0.48 mmol) were added, and the mixture was refluxed overnight. The mixture was brought to room temperature, and volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent afforded the desired product as yellow solid (0.14 g, 67%): R_f = 0.66 (dichloromethane-methanol 8:2 v/v); mp 262 ^{o}C; IR (neat) cm^{-1} 3435, 2937, 2851, 2123, 1735,

1608, 1457, 1241, 1004, 836; ^{1}H NMR (500 MHz, methanol–d_4) δ 7.99 (d, J = 8.83 Hz, 2H), 7.50 (d, J = 8.72 Hz, 1H), 7.15 (d, J = 1.78 Hz, 1H), 7.09-7.05 (m, 3H), 4. 16 (d, J = 2.32 Hz, 2H), 4.08 (t, J = 6.41 Hz, 2H), 3.56 (t, J = 6.58 Hz, 2H), 3.29 (t, J = 4.45 Hz, 4H), 2.87 (t, J = 4.09 Hz, 4H), 2.84 (t, J = 2.14 Hz, 1H), 2.53 (s, 3H), 1.86-1.82 (m, 2H), 1.68-1.62 (m, 2H), 1.58-1.46 (m. 4H); MS (MALDI-TOF) *m/z* calcd. for $C_{27}H_{34}N_4O_2$ 446.26 found 446.44 $[M]^+$;HPLC: t_R 7.60 min, purity 98.4%.

tert-butyl 3-(4-(6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazol-2-yl)phenoxy)propylcarbamate (15). To a solution of 5-(4-methylpiperazin-1-yl)-2-nitroaniline (0.50 g, 2.12 mmol) in a mixture of ethanol-ethyl acetate (20.0 mL, 2:1 *v/v*), Pd-C (0.10 g) was added and the mixture was hydrogenated for 4 h at atmospheric pressure, which afforded the corresponding diamine. Catalyst was filtered off, and the diamine solution was added with *tert*-butyl 3-(4-formylphenoxy) propylcarbamate (0.59 g, 2.12 mmol). To this mixture, an aqueous solution of $Na_2S_2O_5$ (0.40 g, 2.12 mmol in 1.50 mL water) was added, and the mixture was refluxed for 6 h. The reaction mixture was brought to room temperature and the volatiles were removed under reduced pressure. The crude mixture was purified on a silica gel column using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent to afford the desired compound as dark red solid (0.62 g, 63%): R_f = 0.54 (dichloromethane-methanol 8:2 v/v); mp 210–220 ^{o}C; IR (neat) cm^{-1} 2973, 2928, 1678, 1449, 1232, 1175; ^{1}H NMR (500 MHz, DMSO–d_6) δ 12.4 (br, 1H), 8.05 (d, J = 8.5Hz , 2H), 7.42 (br, 1H), 7.07 (d, J = 8.8 Hz, 2H), 6.96-6.91 (m, 2H), 4.05 (t, J = 6.15 Hz, 2H), 3.13 (m, 6H), 2.51 (s, 3H), 1.86 (m, 2H), 1.42 (s, 9H; ^{13}C NMR (125 MHz, methanol–d_4) δ 162.5, 160.1, 156.1, 131.7, 128.0, 123.6, 115.2, 114.6, 77.9,

65.9, 61.8, 55.2, 50.3, 47.6, 46.2, 37.4, 29.6, 28.7, 23.2, 15.6; MS (MALDI-TOF) *m/z*calcd. for $C_{26}H_{35}N_5O_3$465.58, found 465.43 $[M]^+$; HPLC: t_R 6.66 min, purity 97.8%.

Methyl 2-(4-hydroxyphenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole-4-carboxylate (16). To a solution of methyl 3-amino-5-(4-methylpiperazin-1-yl)-2-nitrobenzoate (0.21 g, 0.71 mmol) in a mixture of ethanol-ethyl acetate (15.0 mL, 2:1 v/v), 10% Pd-C (0.06 g) was added and the mixture was hydrogenated for 6 h at room temperature, which afforded the corresponding diamine. Catalyst was filtered off, and the diamine solution was added with 4-hydroxy benzaldehyde (0.09 g, 0.71 mmol). To this mixture, an aqueous solution of $Na_2S_2O_5$ (0.13 g, 0.71 mmol in 0.80 mL H_2O) was added and the mixture was refluxed for 6 h. The reaction mixture was brought to room temperature and the volatiles were removed under reduced pressure. The crude mixture was purified by column chromatography on silica gel using dichloromethane-methanol (0-15% methanol in dichloromethane) as eluent to afford the desired compound as yellow solid (0.13 g, 47%):R_f = 0.50 (dichloromethane-methanol 8:2 *v/v*); mp 260–262 ^oC; IR (neat) cm^{-1} 3460, 2945, 2830, 1715, 1608, 1494, 1273, 1208; ^1H NMR (300 MHz, DMSO–d_6) δ 9.98 (s, br, 1H), 8.10 (d, J = 8.20 Hz, 2H), 7.49-7.45 (2H), 6.90 (d, J = 8.7 Hz, 2H), 3.97 (s, 3H), 3.23 (s, br, 4H), 2.77 (s, br, 4H), 2.42 (s, 3H); ^{13}C NMR (75 MHz, DMSO–d_6) δ 166.2, 159.8, 154.2, 147.1, 146.5, 129.6, 129.2, 121.1, 116.3, 114.4, 114.4, 113.7, 111.3, 55.0, 52.5, 50.1, 45.9; MS (MALDI-TOF) *m/z*calcd. for $C_{20}H_{22}N_4O_3$366.16, found 366.54 $[M]^+$; HPLC: t_R 4.89 min, purity 98.9%.

2-(4-hydroxyphenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole-4-carboxylic acid (17). A solution of methyl 2-(4-hydroxyphenyl)-6-(4-methylpiperazin-1-yl)-1H-benzimidazole-

4-carboxylate (**16**) (55.00 mg, 0.15mmol) in 1 N NaOH (3.00 mL) was heated at 80 ^{o}C for 1 h. The mixture was then cooled to room temperature and neutralized with 1 N HCl. Volatiles were removed, and the crude mixture was purified on a silica gel column to give the desired product as yellow solid (30 mg, 58%): R_f = 0.08 (dichloromethane-methanol 8:2 v/v); mp>260 ^{o}C; IR (neat) cm^{-1} 3362, 2830, 2720, 1723, 1641, 1608, 1494, 1290, 1179, 967; ^{1}H NMR (300 MHz, D_2O) δ 7.48 (d, *J* = 7.9 Hz, 2H), 7.36 (s, br, 1H), 7.09 (s, br, 1H), 6.70 (d, *J* = 8.5Hz, 2H), 3.74-3.71 (br, 2H), 3.60-3.57 (br, 2H), 3.10 (br, 4H), 2.90 (s, 3H); MS (MALDI-TOF) *m/z* calcd. for $C_{19}H_{20}N_4O_3$352.38, found 353.54 $[M+H]^+$; HPLC: t_R 4.29 min, purity 98.1%.

3-(4-(6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazol-2-yl)phenoxy)propan-1-amine (18). To a solution of *tert*-butyl 3-(4-(6-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl) phenoxy) propylcarbamate (**15**) (0.12 g, 0.25 mmol) in dichloromethane (3.00 mL), trifluoroacetic acid (0.50 mL) was added and the mixture was stirred at room temperature for 2h. The volatiles were removed under reduced pressure. The desired compound was obtained in quantitative yield as dark red solid: R_f = 0.03 (dichloromethane-methanol 8:2 v/v); IR (neat) cm^{-1} 3403, 3047, 2953, 1687, 1507, 1266, 1209, 1131, 841, 718; ^{1}H NMR (300 MHz, D_2O) δ 7.74 (d, *J* = 8.7 Hz, 2H), 7.47 (d, *J* = 8.1 Hz, 1H), 7.15-7.02 (m, 4H), 4.06 (t, *J* = 5.50 Hz, 2H), 3.73 (d, *J* = 11.8 Hz, 2H), 3.55 (d, *J* = 11.2 Hz, 2H), 3.14-3.04 (m, 6H), 2.86 (s, 3H), 2.07 (m, 2H); MS (MALDI-TOF) *m/z* calcd. for $C_{21}H_{27}N_5O$ 365.22, found 365.54 $[M+H]^+$; HPLC: t_R 3.22 min, purity 98.2%.

2-(4-((1-(2-azidoethyl)-1H-1,2,3-triazol-4-yl)methoxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole (19). To a solution of **10** (0.10 g, 0.29 mmol) in a mixture of methanol-

water (3.00 mL, 2:1 *v/v*), 1,2 diazido ethane [64](30 fold excess) was added, followed by the addition of a freshly prepared copper catalyst by mixing copper sulfate (0.007 g, 0.043 mmol), sodium ascorbate (0.020 g, 0.100 mmol) in 1.0 mL water. The mixture was stirred overnight at room temperature. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent to afford the desired compounds as pale yellow powder (90 mg, 68%); R_f= 0.40 (dichloromethane-methanol 8:2 v/v); mp 125–135 ^{o}C; IR (neat) cm^{-1} 2099, 1617, 1454, 1229, 1184, 1004; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.18 (s, 1H), 8.01 (d, *J* = 8.78 Hz, 2H), 7.50 (d, *J* = 8.62 Hz, 1H), 7.19 (d, *J* = 8.86 Hz, 1H), 7.14 (d, *J* = 1.72 Hz, 1H), 7.05 (dd, J_1 = 8.61 Hz, J_2 = 1.89 Hz, 1H), 5.29 (s, br, 2H), 4.62 (t, *J* = 5.68 Hz, 2H), 3.85 (t, *J* = 5.40 Hz, 2H), 3.26 (t, *J* = 4.29 Hz, 4H), 2.78 (t, *J* = 4.48 Hz, 4H), 2.46 (s, 3H); ^{13}C NMR (75 MHz, methanol-d_4) δ 159.8, 151.5, 148.0, 143.5, 127.7, 124.6, 122.8, 115.0, 61.0, 54.6, 50.3, 50.1, 49.3, 44.4; MS (MALDI-TOF) *m/z*calcd for $C_{23}H_{26}N_{10}O$ 458. 22, found 458.15 $[M]^+$; HPLC: t_R5.54 min, purity 96.8%.

2-(4-(2-(4-(4-ethynylphenyl)-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole (21). To a solution of 2-(4-(2-azidoethoxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole (**11**) (0.08 g, 0.21 mmol) in a mixture of methanol-water (3.00 mL, 2:1 v/v), $CuSO_4$ (0.007 g, 0.043 mmol) and sodium ascorbate (0.020 g, 0.100 mmol) were added. To this, 1,4 diethynylbenzene (57 fold excess) was added and the reaction mixture was stirred at room temperature for 24 h. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent. The desired

compound was obtained as pale yellow powder (0.06 g, 57%); R_f = 0.48 (dichloromethane-methanol 8:2 v/v); mp 198–200 ^{o}C; IR (neat) cm^{-1} 2937, 2798, 2103, 1585, 1437, 1282, 959; ^{1}H NMR (500 MHz, methanol-d_4) δ 8.47 (s, 1H), 7.96 (d, *J* = 8.60 Hz, 2H), 7.83 (d, *J* = 8.25 Hz, 2H), 7.53 (d, *J* = 8.42 Hz, 2H), 7.487 (d, *J* = 8.78 Hz, 1H), 7.10-7.02 (m, 4H), 4.89 (t, *J* = 4.91 Hz , 2H), 4.53 (t, *J* = 4.91 Hz, 2H), 3.58 (s, 1H), 3.22 (t, *J* = 4.27 Hz, 4H) , 2.72 (t, *J* = 4.38 Hz, 4H), 2.42 (s, 3H) ; ^{13}C NMR (75 MHz, methanol–*d*$_4$) δ 159.6, 151.5, 148.1, 146.6, 140.1, 132.1, 130.6, 127.7, 125.2, 123.0, 122.1, 122.0, 114.9, 114.7,101.1, 82.2, 66.2, 54.6, 50.2, 49.6, 44.; MS (MALDI-TOF) *m/z* calcd for $C_{30}H_{29}N_7O$ 503.24, found 504.23 $[M]^+$;HPLC: t_R 7.12 min, purity 98.0%

6-(4-methylpiperazin-1-yl)-2-(4-(2-(4-(oct-7-ynyl)-1H-1,2,3-triazol-1-yl)ethoxy)phenyl)-1H-benzo[d]imidazole (22). To a solution of 2-(4-(2-azidoethoxy)phenyl)-6-(4-methylpiperazin-1-yl)-1H-benzo[d]imidazole (**11**) (0.08 g, 0.21 mmol) in a mixture of methanol-water (3.00 mL, 2:1 v/v), $CuSO_4$ (0.007 g, 0.043 mmol) and sodium ascorbate (0.020 mg, 0.100 mmol) were added. To this solution, 1,9 decadiyne (1.61 g, 12.0 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. Volatiles were removed under reduced pressure. The crude product was purified by column chromatography using dichloromethane-methanol (0–15% methanol in dichloromethane) as eluent. The desired compound was obtained as pale yellow powder (0.09 g, 79%): R_f = 0.32 (dichloromethane-methanol 8: 2 *v/v*); mp 85–86 ^{o}C; IR (neat) cm^{-1} 2931, 2852, 2114, 1636, 1606, 1451, 1289, 1248, 1173, 1052, 965; ^{1}H NMR (500 MHz, methanol–*d*$_4$) δ 7.97-7.95 (d, *J* = 8.70 Hz, 2H), 7.81 (s, 1H), 7.48 (d, *J* = 8.70 Hz, 1H), 7.11 (d, *J* = 1.34 Hz, 1H), 7.04-7.01 (m, 3H), 4.78 (t, *J* = 4.68 Hz, 2H), 4.45 (t, *J* = 4.68 Hz, 2H), 3.21 (t, *J* = 4.35 Hz, 4H), 2.69-2.66 (m, 6H), 2.38 (s, 3H), 2.17 (t, *J* = 2.68 Hz, 1H),

2.11-2.08 (m, 2H), 1.67-1.61 (m, 2H), 1.48-1.28 (m, 8H); ^{13}C NMR (125 MHz, methanol–d_4) d 159.7, 151.4, 148.1, 147.8, 127.7, 123.0, 122.5, 114.9, 114.7, 83.6, 68.1, 66.3, 54.2, 50.3, 49.4, 44.6, 29.0, 28.1, 28.0, 24.7, 17.5; MS (MALDI-TOF) *m/z* calcd for $C_{30}H_{37}N_7O$ 511.30, found 511.40;HPLC: t_R 7.30 min, purity 97.1%.

Ultra Violet (UV) Thermal Denaturation Experiments. All UV spectra were obtained on a 12 cell holder Cary 1E UV-Vis spectrophotometer equipped with temperature controller. Quartz cells with 1 cm path length were used for all the experiments. Spectrophotometer stability and wavelength alignment were checked prior to initiation of each thermal denaturation experiment. For all experiments, the samples were prepared by diluting a stock sample. The melting of DNA with and without the compounds was performed at a heating rate of 0.2 °C/min. Samples were brought back to 20 °C after the experiment. All UV melting experiments were monitored at 260 nm. For the T_m determinations, derivatives were used. Data points were recorded every 1.0 °C. The DNA concentration was 1 μM /duplex while the compound concentration was 10 μM. All compound solutions were prepared in dimethyl sulfoxide (DMSO) as concentrated stock solution and diluted to desired concentrations in buffer.

Minimum Inhibitory Concentration (MIC) Determination.

Bacteria used in this study were *Staphylococcus aureus* ATCC 29213*, Escherichia coli* ATCC 25922, *Escherichia coli* K12, *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus* MRSA33591, *Staphylococcus aureus* MRSA A960649, *Staphylococcus epidermidis* ATCC 12384, *Acinetobacter baumanni* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* NR15410, *Citrobacter freundii* 4747CFAA, *Shigella flexneri*

2457NR517, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecium* BM4105RF, and *Enterococcus faecalis* ATCC 29212. All compounds were tested by microbroth dilution method following Clinical and Laboratory Standards Institute guidelines.[65] Briefly, Mueller-Hinton broth (Difco Laboratories, Becton Dickinson) was inoculated with each organism and incubated at 37 °C with shaking to establish logarithmic growth. Following incubation, each culture was pelleted by centrifugation (3,500 x g for 5 min) and resuspended in 0.85% sterile saline solution to an optical density of 0.1 at 625 nm. Samples were tested in triplicate using 96 well microplates (Corning Costar Corp. Cambridge, MA), yielding final bacterial concentrations of 5×10^5 CFU/mL, and incubated for 24 h at 37 °C. Following incubation, optical densities of each well were determined with a μQuant microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT) at 625 nm. The MIC was defined as the lowest concentration needed to completely inhibit growth as compared to no treatment controls.

Topoisomerase I Inhibition Assay. The *E. coli* DNA topoisomerase I inhibition assay was used to determine the activities of the newly synthesized compounds against *E. coli* topoisomerase I. The reaction mixture (30 μl) contained 20 mM Tris-HCl at pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 1 μg/mL BSA, 150 ng supercoiled plasmid pBAD-GFPuv, 6 nM of *E. coli* topoisomerase I, and one of the drugs at a specified concentration that ranges from 0.5 to 45 μM. All components were assembled on ice and incubated for 15 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with ethidium bromide, destained, and photographed under UV light. The

intensity of DNA topoisomers was determined using KODAK 1D Image analysis software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was still in a (-) supercoiled state.

Inhibition assays against *E. coli* DNA gyrase. The reaction mixture (30 μL) contained 35 mM Tris-HCl at pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 1.75 mM ATP, 5 mM spermidine, 0.1 mg/ml BSA, 6.5% glycerol, 250 ng of the relaxed plasmid pBAD-GFPuv, 0.5 units of *E. coli* DNA Gyrase, and one of the compounds at a final concentration that ranges from 1 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with ethidium bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software.

Inhibition assays against human DNA topoisomerase I. The inhibition assays were used to determine the activities of the tested compounds against Human DNA topoisomerase I. The reaction mixture (25 μL) contained 20 mM Tris-HCl at pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 1 μg/ml BSA, 250 ng of the supercoiled plasmid pBAD-GFPuv, two units of Human DNA topoisomerase I, and one of the test compounds at a final concentration that ranges from 5 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an

equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer at pH 7.8. Following electrophoresis, the agarose gel was stained with ethidium bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D Image Analysis Software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was in a (-) supercoiled state.

Inhibition Assays against human DNA topoisomerase II. The reaction mixture (25μL) contained 20 mM Tris-HCl at pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 1 μg/mL BSA, 200 ng supercoiled plasmid pBAD-GFPuv, 4 units of Human topoisomerase II, and one of the test compounds at a final concentration that ranges from 5 to 50 μM. All components were assembled on ice and incubated for 30 min at 37 °C. After the incubation, the reactions were terminated by extraction with an equal volume of phenol. The topological state of the DNA samples was analyzed with 1% agarose gel electrophoresis in 1×TAE buffer, pH 7.8. Following electrophoresis, the agarose gel was stained with ethidium bromide, destained, and photographed under UV light. The intensity of DNA topoisomers was determined using KODAK 1D image analysis software. The percentage of (-) supercoiled DNA was calculated from a comparison of the intensity of the (-) supercoiled band with the total intensity of all DNA topoisomers. The IC_{50} was calculated as the amount of the drug for which 50% of the DNA was in a (-) supercoiled state.

RNA topoisomerase inhibition: RNA topoisomerase assay was performed as described earlier.[51, 66] To inhibit the RNA topoisomerase activity, all inhibitors were diluted in DMSO. *E.*

coli topoisomerase I, along with the reaction buffer was first mixed with each specific inhibitor. The circular RNA substrate was then added to the reaction mixture and incubated for 1.5 h. The reaction was terminated using stop buffer containing proteinase K, SDS and EDTA. [51, 66] The reaction product, a trefoil RNA knot, was distinguished from the circular RNA substrate on 15% TBE-urea gels (Invitrogen), and analyzed by Storm 860 Molecular Imager (Molecular Dynamics).

Docking experiments: Molecular docking was performed over PatchDock web server (http://bioinfo3d.cs.tau.ac.il/PatchDock),[67] an online tool for protein docking designed for the purpose of the identification of the interaction sites between *E. coli* topoisomerase I-ssDNA complexes with the most potent ligands **3** and **6**, wherein the molecular surface of the protein/enzyme is divided into patches as per the molecular shape followed by the comparison between the patches in order to produce a group of transformations. Prepared PDB files of ligands and receptors were provided to PatchDock server at default value of 4.0 Å for clustering RMSD and default complex type. The PDB coordinates of *E. coli* Topoisomerase I complex (PDB ID. 3PX7) were taken from Protein Data Bank and prepared for dockings by deleting undesired protein chains and ligand. A wide interface is ensured to include several matched local features of the docked molecules that have complementary characteristics. These transformations are then ranked as per the geometric complementarity score and each transformation is assigned with a PatchDock score as well as atomic contact energy. Docking with Patchdock was validated with other Docking softwares available from online servers e.g. Swiss Dock, ZDOCK and Dock Blaster. Accelrys Discovery Studio[68] was used for structural analysis for all docked complexes. Final figures were made using PyMOL (https://www.pymol.org/) and Accelrys Discovery Studio Visualizer.

Cytotoxicity Studies. DU 145 and PCS 201-010 cell lines were cultured according to ATCC protocols. Cells were harvested using trypsin–EDTA solution and counted using trypan blue exclusion. Cells were seeded at a volume of 100 mL per well in the wells of tissue culture treated 96 well plates at a density of 10×10^5 cells per well. Seeded 96 well plates were returned to incubator (37 ºC, 5% CO_2) for twenty-four hours to resume exponential growth. Test compounds (Compound **1-9**) and a control compound (Hoechst 3328) were diluted in appropriate culture media to the following concentrations: 40, 20, 10, 5, 2.5, 1.25 and 0.125 μM. Cell lines were then treated with 100 mL of each test compound or control in triplicate. The final concentrations of each treatment were: 20, 10, 5, 2.5, 1.25, 0.625 and 0.0625 μM. Each plate also contained wells containing untreated cells and media only as controls. After receiving treatments, the 96 well plates were returned to incubator (37 ºC, 5% CO_2) for forty-eight hours. After forty-eight hours of treatment, the treated plates were fixed with trichloroacetic acid and stained with sulforhodamine B using a modified version of the protocol described by Skehan et al.[69] In short, 50 mL of cold 80% TCA was added to each well, at a final concentration of 16% TCA, and plates were incubated at 4 ºC for one hour. The media and TCA solution was discarded and plates were washed four times using room temperature tap water. Plates were allowed to air dry overnight. Plates were stained with the addition of 70 mL per well of 40% (w/v) SRB in 1% (v/v) acetic acid solution. Samples were stained for fifteen minutes and then stain was discarded. Plates were then washed four times with 1% (v/v) acetic acid solution to remove unbound stain and allowed to air dry overnight at room temperature. Finally, SRB stain was solubilized by adding 150 mL of 10 mM unbuffered Tris base to each well. The absorbance of each samples at 560 nm was recorded using a Tecan plate reader and the IC_{50} was determined using Origin 5.0 software. Each study was completed in duplicate.

HPLC analysis. HPLC analysis of compounds **5-22** was performed on HP1100 series analytical HPLC instrument. The experiments were performed on a Supelcosil LC-18S column using the following gradient conditions-Compounds **5-9**: 60% B in A with initial hold for 2 minutes and then equilibrate at 60% B in A to 100% B over 8 minutes at a flow rate of 2.0 mL/minute; compounds **10-22**. 0-100% B in A over 10 minutes at a flow rate of 2.0 mL/minute; where, A- H_2O containing 0.1% trifluoroacetic acid and B- acetonitrile.

ASSOCIATED CONTENT

Supporting Information. UV thermal denaturation profiles, ^{1}H NMR, ^{13}C NMR, IR, HPLC and MALDI-TOF spectra of all new compounds and DNA topoisomerase I inhibition assays, Molecular Formula Strings file (.CSV) . This information is available free of charge via the internet at http://pubs.acs.org.

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CONFLICT OF INTEREST STATEMENT

DPA has ownership interest in NUBAD LLC.

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ABBREVIATIONS

UV, Ultra Violet; NMR, Nuclear Magnetic Resonance; TLC, thin layer chromatography; MIC, Minimum Inhibitory Concentration

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