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Remarkable G2/M phase arrest and apoptotic effect performed by 2-(6-aryl-3-hexen-1,5-diynyl) benzonitrile antitumor agents

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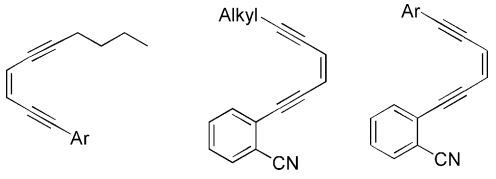
Abstract—2-(6-aryl-3-hexen-1,5-diynyl)benzonitriles **3a–j** showed growth inhibition effects on a full panel of 60 human cancer cell lines in low micro-concentrations, in which compounds **3c**,**d** displayed a significant G2/M arrest in the cell growth cycle compared with other derivatives and an apoptotic progress induction were also shown by **3a–d**.

1. Introduction

Natural and synthetic components containing enediyne subdomain cores, which were either isolated or derived from Streptomyces, display unique biological activities[1,2] which are sourced from a specific generation mode of diradical intermediates. Except for formation of active radicals, enediynes have received limited attention in other feasible physiological active modes, and there have been no investigations describing related phenomena, although some structure–activity relationships (SAR) were explored in our previous work.[3] However, precise knowledge of the relationship between cytotoxicities and active mechanisms of these unique structures are still under investigation, and to achieve the goal, further study of these novel enediynes is necessary.

In our most recent report, several series of acyclic enediynes, 1-aryl-3-decen-1,5-diynes **1**,[3a] exhibited cytotoxicities toward KB, Hela, DLD, NCI and Hepa cell lines in low range of micromolar concentrations. 2-(6-alkyl-3-hexen-1,5-diynyl)benzonitrile **2**[3b] also showed selective potent effect in growth inhibition of Hepa cell line. It was considered that compounds with an aryl substituent on the C-6 position displayed higher cytotoxicity than alkyl compounds on that position. To discover more evidence of the biological activities of these novel enediynes, 2-(6-aryl-3-hexen-1,5-diynyl) benzonitriles **3a–j** were designed and synthesized by modification of the aryl groups on the C-6 position of the enediyne core. These derivatives were evaluated for cytotoxic responses against sixty human tumor cell lines,[5] and the cell cycle analysis was performed to provide more advanced understanding of their cytotoxicities.



1 2 3

2. Results

2.1. Chemistry

2-(6-Aryl-3-hexen-1,5-diynyl)benzonitriles **3a–j** were prepared from 1-chloro-4-trimethylsilyl-1-buten-3-yne **4**[3a] as the starting material (Scheme 1). Sonogashira coupling reaction[4] with 2-ethynylbenzonitrile **5**[3a] gave **6** and subsequently treatment of **6** with tetrabutylammonium fluoride (TBAF) provided 2-(3-hexen-1,5-diynyl)benzonitrile **7** in 53% yield. Palladium-catalyzed coupling reaction with various aryl iodides **8a–j** gave **3a–j** in 35–90% yields, respectively.

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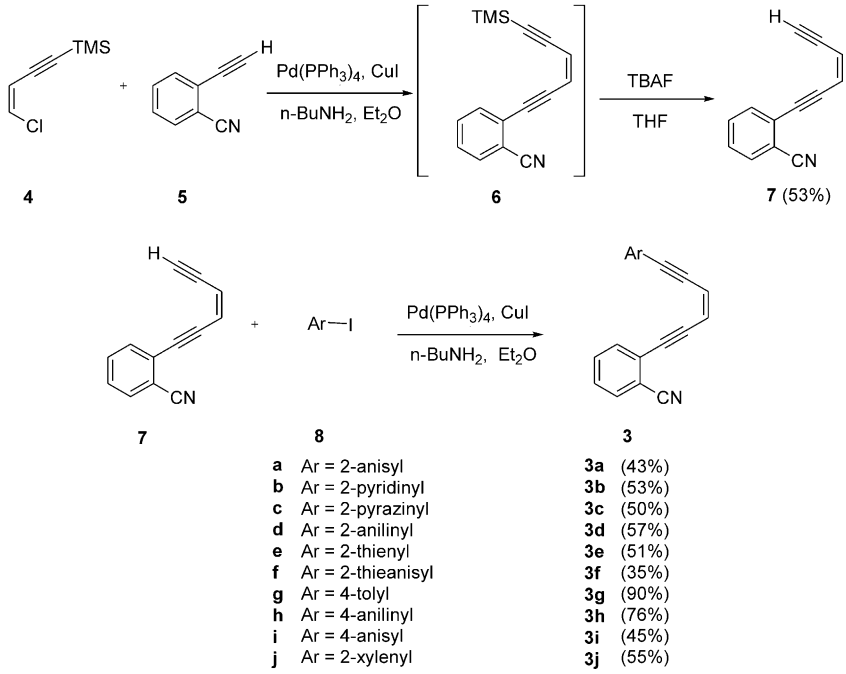


Scheme 1.

3. Cytotoxicity

Compounds **3a–j** were submitted to the National Cancer Institute for testing against a panel of approximately 60 tumor cell lines. Details of this test system have been published by others.[6] Compounds **3e–j** were inactive.[7] The available IC_{50} values of **3a–d** were summarized in Table 1. Obviously, compounds **3a–d** displayed a broad-spectrum inhibition on the growth of all 60 cancer cell lines, and most of the average IC_{50} values of **3a–d** were from 0.43 μM to 33.6 μM. Among the four active compounds, 2-(6-(2-anilinyl)-3-hexen-1,5-diynyl) benzonitrile **3d** showed the highest cytotoxic activity against all sixty tumor cell lines at low 10^{-7} M concentration, especially against the MDA-MB-435 cell line of human breast cancer (0.11 μM). It was noted that most of the LC_{50} values of **3a–d** for the sixty cancer cell lines were higher than 10^{-4} M.

3.1. Cell cycle analysis of compounds 3a–d

To obtain much more insight regarding the variances of the enediyne in affecting whole cells, human leukemia K-562 cell was used, and the growth characteristics of cells following treatment with compounds **3a–d** were measured (Fig. 1). As shown in Figure 1 (exchanged to Figs 2 and 3), cancer cells were exposed to the vehicle solvent (DMSO) as control, and 50 μM of **3a–d** were added to the cell line. After exposure to the compounds for 72 h, attached cells were analyzed by flow cytometry. The majority of control cells exposed to DMSO were in either the G0/G1 phase (39.3%) or S phase (43.6%) of the cell cycle and only a few cells in the G2/M phase were detected (17%) (Fig. 2). After treatment with compounds **3c** and **3d** for 72 h, cells progressed to the G2/M phase, and the majority of the cell population was arrested at the G2/M phase. Consistent with this cell cycle, only 13.7% and 6.4% of the cells were found at the G0/G1 phase, and 9% and 9.6% in S phase, with 77.3% and 84.3% in G2/M phase.

Compounds **3a**,**b** showed moderate accumulation of G2/M phase cells, which were 28% and 39.7%. However, remarkable blockage of the K-562 cell cycle in the G2/M phase was observed and induced at the concentration (50 μM) of compounds **3c** and **3d**. On the other hand, compounds **3a–d** induced significant apoptosis of K-562 cells at a concentration of 50 μM. The percentages of apoptosis for **3a–d** and the control were as following: **3a** (21.3%), **3b** (22.9%), **3c** (29.7%), **3d** (24.2%) and the control (11.5%). Component **3c** had the highest induction of apoptosis (29.7%) after treating K-562 cells with the compound for 72 hr (Fig. 3). However, **3c** did not show the highest cytotoxic activity for the K-562 cell line.

4. Discussions

There were several points of interest arising from this study and the conclusions were summarized in two parts. (1) For the cytotoxic assay, (a) Compounds **3a–d** displayed a greater growth inhibitory activies compared with other derivatives. It was considered that the essential factors for showing the inhibitory activities on human cancer cells were either containing the N atom in the aryl ring, or bearing NH_2 or OCH_3 groups closed to the C-6 position of the enediyne cores, which probably provided a hydrogen bonding or coordination with metal ions cooperating with the central enediyne. Compound **3d** offered the highest cytotoxicity suggested that the anilinyl subdomain existed stronger binding with the unknown target than other derivatives. (b) The growth inhibition effects of nine common kinds of human

Table 1. The in vitro testing results of full panel screen of sixty human tumor cell lines[a]

Panel/cell line	Cytotoxicity (GI_{50}[b]/LC_{50}[c] in μM)			
	3a	**3b**	**3c**	**3d**
Leukemia				
CCRF-CEM	5.26/>100.0	36.50/>100.0	5.60/>100.0	0.32/>100.0
HL-60 (TB)	4.55/>100.0	6.71/>100.0	3.97/>100.0	0.38/>100.0
K-562	3.68/>100.0	4.07/>100.0	4.88/>100.0	0.49/>100.0
MOLT-4	0.27/>100.0	NT	5.93/>100.0	0.51/>100.0
RPMI-8226	NT	NT	5.60/>100.0	0.64/>100.0
SR	4.09/>100.0	4.12/>100.0	4.30/>100.0	0.25/>100.0
Non-Small Cell Lung Cancer				
A549/ATCC	6.89/>100.0	19.80/>100.0	37.10/>100.0	0.87/>100.0
EKVX	NT	22.90/>100.0	36.20//>100.0	2.26/>100.0
HOP-92	31.70/>100.0	26.90/>100.0	>100.00/>100.0	5.00/>100.0
NCI-H226	NT	NT	21.90/>100.0	0.71/>100.0
NCI-H23	5.61/>100.0	12.30/52.6	15.90/74.2	0.48/>100.0
NCI-H322M	NT	NT	68.20/>100.0	3.44/>100.0
NCI-H460	13.30/>100.0	20.40/96.3	28.90/>100.0	0.56/>100.0
NCI-H522	5.14/>100.0	15.90/84.7	14.80/>100.0	3.26/8.6
Colon Cancer				
COLO 205	13.30/>100.0	27.40/>100.0	40.90/>100.0	0.93/>100.0
HCC-2998	6.20/>100.0	14.50/52.6	22.40/93.1	0.26/8.2
HCT-15	6.94/>100.0	18.80/>100.0	16.20/85.4	0.39/42.6
HT29	6.28/>100.0	18.00/95.8	13.40/>100.0	0.55/>100.0
KM12	6.37/>100.0	10.90/80.4	43.90/>100.0	0.34/>100.0
SW-620	6.33/>100.0	12.6080.5	69.80/>100.0	0.40/>100.0
CNS Cancer				
SF-268	19.40/>100.0	16.10/>100.0	26.20/>100.0	1.63/>100.0
SF-295	6.57/>100.0	20.10/>100.0	17.60/>100.0	0.25/12.4
SF-539	18.6/>100.0	20.50/98.9	17.60/>100.0	0.51/>100.0
SNB-19	37.70/>100.0	25.90/>100.0	41.00/>100.0	1.91/>100.0
SNB-75	28.70/>100.0	24.80/>100.0	54.60/>100.0	0.94/>100.0
U251	9.17/>100.0	18.50/79.0	26.10/>100.0	0.43/>100.0
Melanoma				
LOX IMVI	NT	NT	4.44/>100.0	0.45/68.2
MALME-3M	9.98/>100.0	19.30/81.3	24.10/>100.0	0.42/>100.0
M14	6.08/>100.0	15.40/53.7	19.50/>100.0	0.24/47.3
SK-MEL-2	14.90/>100.0	14.90/74.0	8.34/>100.0	1.17/>100.0
SK-MEL-28	34.20/>100.0	17.50/73.8	35.50/>100.0	2.27/>100.0
SK-MEL-5	2.09/23.3	2.99/41.6	19.60/>100.0	0.44/33.1
UACC-257	5.20/>100.0	13.70/60.3	21.40/>100.0	0.78/>100.0
UACC-62	23.70/>100.0	15.3/89.7	16.70/>100.0	0.38/63.0
Ovarian cancer				
IGROV1	10.40/>100.0	17.00/79.1	46.20/>100.0	0.27/>100.0
OVCAR-3	4.40/>100.0	4.26/56.9	16.60/>100.0	0.17/9.8
OVCAR-4	96.90/>100.0	75.80/>100.0	46.50/>100.0	3.85/>100.0
OVCAR-5	39.20/>100.0	28.60/>100.0	35.20/>100.0	0.86/>100.0
OVCAR-8	5.34/>100.0	13.10/70.0	3.11/>100.0	1.22/>100.0
SK-OV-3	39.80/>100.0	25.10/>100.0	52.70/>100.0	3.22/>100.0
Renal Cancer				
ACHN	13.90/>100.0	29.60/>100.0	35.70/>100.0	1.16/>100.0
CAKI-1	3.73/>100.0	21.80/>100.0	21.60/>100.0	0.13/35.9
RXF 393	7.98/>100.0	14.10/77.3	13.70/>100.0	0.80/>100.0
SN12C	26.00/>100.0	20.40/>100.0	22.60/>100.0	0.41/52.4
TK-10	20.80/>100.0	30.90/>100.0	24.00/>100.0	0.84/>100.0
UO-31	10.90/>100.0	15.30/>100.0	39.70/>100.0	0.64/>100.0
Prostate Cancer				
PC-3	13.20/>100.0	13.70/>100.0	30.90/>100.0	2.78/>100.0
DU-145	2.10/>100.0	28.60/>100.0	36.30/>100.0	3.48/>100.0
Breast Cancer				
MCF7	4.61/>100.0	14.30/78.2	36.00/>100.0	0.43/>100.0
NCI/ADR-RES	10.40/>100.0	16.50/71.0	15.90/>100.0	0.38/>100.0
MDA-MB-231/ATCC	NT	NT	23.10/>100.0	0.28/61.2
HS 578T	25.90/>100.0	16.50/89.1	33.10/>100.0	0.83/>100.0
MDA-MB-435	3.23/>100.0	3.52/>100.0	5.30/>100.0	0.11/0.7
BT-549	NT	NT	20.30/>100.0	1.01/>100.0
T-47D	5.86/>100.0	20.80/>100.0	>100.0/>100.0	2.97/>100.0

[a] Data obtained from the NCI's in vitro human tumor cell screen.
[b] The concentration produces 50% reduction in cell growth.
[c] The concentration produces 50% cells kill.

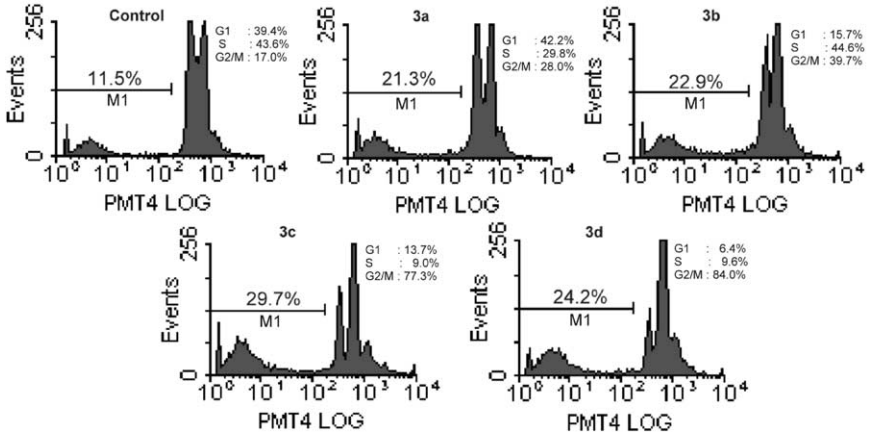


Figure 1. Cell cycle distribution of K-562 cells treated with the control and **3a–d** for 72 h at a concentration of 50 μM by flow cytometry analysis. M1 = apoptotic sub-G1 area.

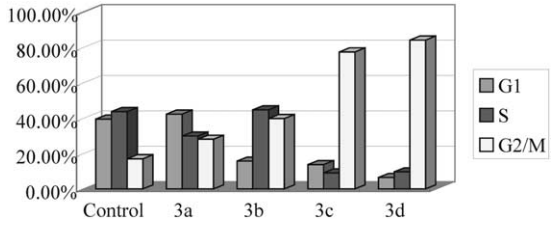


Figure 2. Cell cycle distribution of K-562 cells after treating with compound **3a–d** and DMSO. *The percentages of the cells in each phase were calculated by using the WinMDI software for the flow cytometry. The percentages of accumulation of G2/M phase cells of the control and compounds **3a–d** were 17%, 28%, 39.7%, 77.3% and 84.0%, respectively.

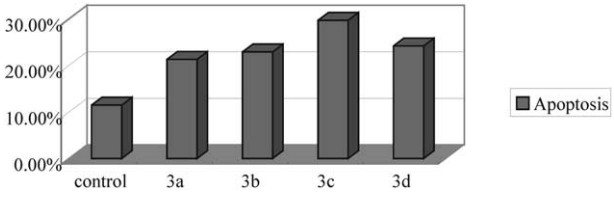
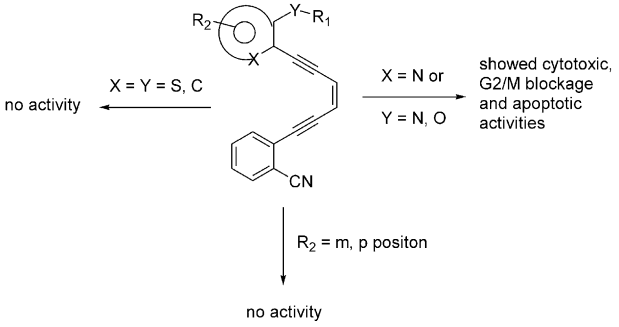


Figure 3. Apoptotic effect induced by compounds **3a–d**. *The percentages of the cells apoptosis were calculated by using the WinMDI software for the flow cytometry. The proportions of apoptotic cells for compounds **3a–d** and the control were: **3a** (21.3%), **3b** (22.9%), **3c** (29.7%), **3d** (24.2%) and the control (11.5%).

tumor cells, compared by the levels of average IC_{50} (μM), were: **3d** > **3a** > **3b** ≒ **3c**. (c) Most of the demonstrated LC_{50} values of compounds **3a–d** for the sixty cancer cell lines were higher than 100 μM, which suggested that analogues **3a–d** displayed growth inhibitory activities to human tumor cells, and caused neither normal nor cancer cells' deaths even when the concentrations of these drugs were as high as 100 μM. This might possibly do some help in more understanding to the major drawback of the applications of anticancer drugs in clinical therapies, the serious side-effects due to the large number of normal functional cells destroyed by the increasing dosages of antitumor agents. This phenomenon was unusual and meaningful to the advancement of medical therapies of human cancer diseases, whereas drugs with higher cytotoxic activities always followed the higher damage to normal cells (lower LC_{50} values). (2) For cell cycle assay, according to the data shown in Figures 2 and 3, when K-562 cells were treated with compounds **3a–d** for 72 h, it was demonstrated that an accumulation of G2/M stage cells in the **3c**,**d** samples. The percentage of cells at the G2/M phase increased from 17% to 77.3% and 84% after treatment of K-562 cells with **3c** and **d**. Similar G2/M blockage was also induced by compounds **3a**,**b**. It was assumed that G2/M phase arrest was possible due to the inhibition of enzymes essential for G2/M progression or mitosis. On the other hand, all four active structures caused the formation of apoptotic K-562 cells at 50 μM, and the proportion of apoptotic cells were from 21.3% to 29.7%. Generally, there are two types of cellular death, and that are necrosis and apoptosis.[8] Necrotic cells are killed by external or tumor necrotic factors (TNF), while apoptotic cells participated in their own destruction. The presentation of apoptotic effect excluded the TNF for providing cytotoxicities to cancer cells in the presence of **3a–d**. Usually the apoptotic process was very complex. An important part of the phenomenon could be mediated by deregulation in cell cycle progression governed by a family of cyclin-dependent kinases (CDKs), although much more experimental evidences were necessary to support this hypothesis. However, compounds **3a–d** led K-562 cells to apoptosis, and **3c**,**d** displayed a significant G2/M arrest in the cell growth cycle.

5. Conclusions

Based upon the above results of cytotoxicity and cell cycle assay of compounds **3a–j**, a preliminary picture about the structure–activity relationship could be achieved (shown in Scheme 2). It was found that derivatives **3a–d** showed more potent biological activities than other derivatives during the evaluation course. The profiles were suggested that the biological activities of these compounds were sourced from the appearance of heteroatoms (N, O) closed to the C-6 position of enediyne domains. It was thought that the necessity of basic heteroatoms (N, O) could be due to the formation of hydrogen binding between the heteroatoms and the target enzymes in addition to the complexation of the enzymes with enediyne cores. This could also explain



Scheme 2. The structure–activity relationship of 2-(6-aryl-3-hexen-1,5-diynyl)benzonitriles.

why the less basic heteroatom (S) containing compounds **3e** and **f** were less active. Although the actual mechanism of **3a–d** is not clear, the inhibitors of essential enzymes of topological and mitosis were considered, however, more evidences are necessary to support this prediction.

In brief, this study has revealed several specific G2/M phase blocker lead compounds together with an apoptotic progress induction, and has showed growth inhibition effects on a full panel of 60 human cancer cell lines in low micro-concentrations. These new investigations will be helpful in further elucidation of undiscovered biological properties of these novel antitumor enediynes and the latest report will be released when the greater approaches are available.

6. Experimental

6.1. General procedure for coupling (2-(3-hexen-1,5-diynyl) benzonitrile 7 with various aryl iodides

To a degassed solution of (2-(3-hexen-1,5-diynyl) benzonitrile (**7**) (12 mmol) in Et_2O (25 mL) containing CuI (3.2 mmol) and *n*-$BuNH_2$ (34 mmol) in Et_2O (25 mL) was added a degassed solution of aryl iodides (**8**) (12 mmol) containing $Pd(PPh_3)_4$ (0.8 mmol) in Et_2O (25 mL). The resulting reaction mixture was stirred for 6 h and quenched with saturated aqueous NH_4Cl solution. The aqueous layer was extracted with EtOAc (50 mL) and the combined organic extracts were washed with saturated aqueous Na_2CO_3 solution (40 mL) and dried over anhydrous $MgSO_4$. After filtration and removal of solvent in vacuo, the residue was purified by column chromatography on silica gel to yield the desired products.

6.1.1. General procedure of the desilylation reaction by using TBAF. To a degassed solution of 2-(6-trimethylsilyl-3-hexen-1,5-diynyl)benzonitriles **6** (1 mmol) in dry THF (15 mL), TBAF (1.2 mmol) was added to the solution and stirred for 6 h at 25 °C, quenched with saturated aqueous NaCl solutions and extracted with EtOAc. The organic layer was separated and dried over $MgSO_4$. After filtration, the solvent was evaporated in vacuo. The residue was purified by flash chromatography to give the product **7**.

6.1.2. Cell cycle analysis. Samples of 1×10^6 K-562 cells were plated on a 6 cm tissue culture dish, and the cells were allowed to recover 24 h before any further treatment. DMSO or compound **3a–d** (50 μM) were then added to the cells for 1 h in complete medium at 37 °C, washed twice with PBS and incubated in fresh media for the incubation time. Cell were harvested with trypsin and washed twice with PBS. Samples were fixed in 70% ethanol and stored at 4 °C for at least 24 h, then washed once with McIlvaine's buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH = 7.5) and once again with PBS. Samples were stained with PI (propidium iodide) staining solution (PBS containing 100 μg/mL, RNase A and 10 μg/mL PI [sigma]), processed on a Coulter Elite flow cytometry, and analyzed using the Mutiplus AV program.

6.1.3. 2-(3-Hexen-1, 5-diyne) benzonitrile (7). As a brown oil in 53% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.67 (dd, 1H, J = 1.6, 0.4 Hz), 7.65–7.53 (m, 2H), 7.42 (td, 1H, J = 7.8, 1.2 Hz), 6.20 (dd, 1H, J = 6.8, 0.8 Hz), 5.98 (dd, 1H, J = 11.2, 2.4 Hz), 3.52 (dd, 1H, J = 2.4, 0.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 132.8, 132.8, 132.3, 128.7, 126.6, 120.6, 120.5, 117.2, 115.0, 92.7, 92.3, 86.7, 80.3. MS (EI) [m/z (relative intensity)]: 177 (M^+, 100), 162 (31), 161 (94), 150 (76). HRMS: calcd for $C_{13}H_7N$: Mr = 117.0579. Found, 117.0579.

6.1.4. 2-(6-(2-Anisyl)-3-hexen-1, 5-diyne) benzonitrile (3a). Obtained as a brown oil in 40% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.64–7.62 (m, 2H), 7.54 (td, 2H, J = 7.6, 1.2 Hz), 7.40 (td, 1H, J = 7.6, 1.2 Hz), 7.34–7.30 (m, 1H), 6.95–6.88 (m, 2H), 6.28 (d, 1H, J = 10.8 Hz), 6.14 (d, 1H, J = 10.8 Hz), 3.84 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 160.1, 134.1, 132.9, 132.8, 132.2, 130.5, 128.5, 127.2, 122.1, 120.6, 117.6, 117.4, 115.0, 112.2, 110.8, 95.4, 93.5, 92.7, 91.0, 55.9. MS (EI) [m/z (relative intensity)]: 283 (M^+, 100), 282 (72), 254 (21), 240 (14) 238 (14) 181 (21). HRMS: calcd for $C_{17}H_9NS$: Mr = 283.0998. Found, 283.0998.

6.1.5. 2-(6-(2-Pyridinyl)-3-hexen-1, 5-diyne) benzonitrile (3b). Obtained as a brown oil in 50% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.64–8.62 (m, 1H), 7.74–7.66 (m, 4H), 7.57 (td, 1H, J = 8.0, 0.8 Hz), 7.43 (td, 1H, J = 8.0, 0.8 Hz), 7.31–7.27 (m, 1H), 6.26 (s, 2H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 149.9, 142.8, 136.0, 132.8, 132.5, 132.2, 128.6, 127.6, 126.5, 123.0, 120.6, 119.7, 117.2, 114.7, 97.1, 93.4, 92.6, 86.0. MS (EI) [m/z (relative intensity)]: 254 (M^+, 100), 252 (69), 227 (8), 226 (6). HRMS: calcd for $C_{18}H_{10}N_2$: Mr = 254.0845. Found, 254.0844.

6.1.6. 2-(6-(2-Pyrazinyl)-3-hexen-1, 5-diyne) benzonitrile (3c). Obtained as a brown oil in 50% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 8.81 (d, 1H, J = 1.2 Hz), 8.58–8.56 (m, 1H), 8.48 (d, 1H, J = 2.2 Hz), 7.65–7.52 (m,3H), 7.47–7.39 (m 1H), 6.32 (d, 1H, J = 11.0 Hz), 6.24 (d, 1H, J = 11.0 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 148.2, 144.4, 143.0, 139.9, 132.8, 132.7, 132.3, 128.9, 126.4, 121.2, 119.9, 117.2, 115.1, 94.1, 94.0, 92.3, 89.9, 87.5. MS (EI) [m/z (relative intensity)]: 256 ($M^+ + 1$, 100), 255 (M^+, 40), 202 (47), 201 (35), 175 (77), 127 (42), 87 (31). HRMS: calcd for $C_{17}H_9N_3$: Mr = 255.0798. Found, 255.0804.

6.1.7. 2-(6-(2-Anilinyl)-3-hexen-1, 5-diyne) benzonitrile (3d). Obtained as a brown oil in 53% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 7.69–7.52 (m, 3H), 7.49–7.22 (m, 2H), 7.19–7.10 (m, 1H), 6.73–6.65 (m, 2H), 6.28 (d, 1H, J = 11.0 Hz), 6.14 (d, 1H, J = 11.0 Hz), 4.35 (bs, 2H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 147.6, 132.9, 132.7, 132.3, 132.2, 130.4, 128.5, 126.7, 121.5, 118.2, 117.4, 116.9, 114.9, 114.6, 107.6, 95.4, 93.5, 92.7, 92.5. MS (EI) [m/z (relative intensity)]: 268 (M^+, 98), 267 (46), 266 (87), 241 (34) 183 (100) 167 (24) 166 (69) 149 (43) 129 (28). HRMS: calcd for $C_{19}H_{12}N_2$: Mr = 268.1002. Found, 268.1024.

6.1.8. 2-(6-(2-Thienyl)-3-hexen-1, 5-diyne) benzonitrile (3e). Obtained as a brown oil in 51% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.66 (t, 2H, J = 8.4 Hz), 7.57 (t, 1H, J = 7.8 Hz), 7.42 (t, 1H, J = 7.8 Hz), 7.36–7.32 (m, 2H), 7.02 (dd, 1H, J = 9.2, 3.6 Hz), 6.21 (d, 1H, J = 10.8 Hz), 6.13 (d, 1H, J = 10.8 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) δ: 133.0, 132.7, 132.3, 128.5, 128.3, 127.3, 126.9, 122.7, 121.2, 119.0, 117.6, 117.3, 114.9, 93.1, 91.9, 91.5, 91.0. MS (EI) [m/z (relative intensity)]: 259 (M^+, 100), 258 (16), 257 (8), 227 (10) 214 (16). HRMS: calcd for $C_{17}H_9NS$: Mr = 259.0457. Found, 259.0463.

6.1.9. 2-(6-(2-Thieanisyl)-3-hexen-1, 5-diyne) benzonitrile (3f). Obtained as a brown oil in 35% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.68–7.65 (m, 2H), 7.57–7.50 (m, 2H), 7.42 (dd, 1H, J = 7.6, 2.4 Hz), 7.39–7.32 (m, 1H), 7.17–7.08 (m, 2H), 6.29 (d, 1H, J = 10.8 Hz), 6.19 (d, 1H, J = 10.8 Hz), 2.41 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 142.0, 133.0, 132.9, 132.6, 132.1, 129.2, 129.2, 128.4, 128.4, 127.0, 124.2, 124.1, 121.3, 118.1, 117.3, 95.7, 93.3, 93.0, 92.8, 15.0. MS (EI) [m/z (relative intensity)]: 299 (M^+, 20), 298 (24), 285 (19), 284 (100), 277 (11). HRMS: calcd for $C_{20}H_{13}NS$: Mr = 299.0769. Found, 299.0764.

6.1.10. 2-(6-(4-Tolyl)-3-hexen-1, 5-diyne) benzonitrile (3g). Obtained as a brown oil in 90% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.67 (dd, 1H, J = 8.0, 1.2 Hz), 7.61 (dd, 1H, J = 8.0, 1.2 Hz), 7.56 (td, 1H, J = 8.0, 1.2 Hz), 7.45–7.39 (m, 3H), 7.14 (d, 2H, J = 8.0 Hz), 6.21 (d, 1H, J = 10.8 Hz), 6.12 (d, 1H, J = 10.8 Hz), 2.36 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 139.0, 132.7, 132.7, 132.6, 132.3, 131.8, 131.8, 129.1, 129.1, 128.4, 127.0, 121.8, 119.7, 117.5, 114.9, 99.1, 99.1, 93.2, 92.6, 29.6. MS (EI) [m/z (relative intensity)]: 267 (M^+, 100), 266 (47), 265 (21), 264 (17) 251 (12) 239 (14). HRMS: calcd for $C_{20}H_{13}N$: Mr = 267.1042. Found, 267.1042.

6.1.11. 2-(6-(4-Anilinyl)-3-hexen-1, 5-diyne) benzonitrile (3h). Obtained as a brown oil in 76% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.67–7.64 (m, 1H), 7.61 (dd, 1H, J = 7.6, 1.2 Hz), 7.57–7.55 (m,1H), 7.42–7.40 (m, 3H), 6.63 (dd, 2H, J = 6.6, 1.8 Hz), 6.20 (d, 1H, J = 10.8 Hz), 6.05 (d, 1H, J = 10.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.2, 133.4, 133.1, 132.6, 132.5, 132.2, 132.1, 128.3, 127.0, 123.8, 122.1, 117.4, 115.9, 114.5, 111.8, 100.3, 93.6, 92.2, 85.5. MS (EI) [m/z (relative intensity)]: 268 (M^+, 100), 267 (12), 266 (13), 241 (11) 191 (17). HRMS: calcd for $C_{19}H_{12}N_2$: Mr = 268.1002. Found, 268.1006.

6.1.12. 2-(6-(4-Anisyl)-3-hexen-1, 5-diyne) benzonitrile (3i). Obtained as a brown oil in 45% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.68–7.62 (m, 1H) 7.60 (dd, 1H, J = 12.0, 0.4 Hz), 7.55 (td, 1H, J = 7.6, 1.2 Hz), 7.50 (dd, 2H, J = 6.8, 2.0 Hz), 7.41 (td, 1H, J = 7.8, 1.2 Hz), 6.86 (dd, 2H, J = 6.8, 2.4 Hz), 6.21 (d, 1H, J = 10.8 Hz), 6.10 (d, 1H, J = 10.8 Hz), 3.82 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.0, 133.5, 133.2, 132.7, 132.6, 132.3, 132.0, 128.4, 127.0, 123.5, 121.8, 116.8, 114.9, 114.0, 114.0, 99.2, 93.3, 92.4, 85.9, 55.3. MS (EI) [m/z (relative intensity)]: 283 (M^+, 100), 268 (11), 241 (12), 240 (56) 238 (16). HRMS: calcd for $C_{20}H_{13}ON$: Mr = 283.0998. Found, 283.1003.

6.1.13. 2-(6-(2-Xylenyl)-3-hexen-1, 5-diyne) benzonitrile (3j). Obtained as a brown oil in 55% yield. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.68–7.65 (m, 1H), 7.59–7.53 (m, 2H), 7.13 (d, 1H, J = 7.6 Hz), 7.06 (t, 1H, J = 7.6 Hz), 6.26 (d, 1H, J = 10.8 Hz), 6.15 (d, 1H, J = 10.8 Hz), 2.72 (s, 3H), 2.43 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.8, 136.7, 132.7, 132.5, 132.2, 130.5, 130.2, 128.4, 127.0, 125.3, 122.7, 121.8, 117.4, 115.0, 98.3, 96.8, 93.4, 92.4, 90.2, 20.2, 17.5. MS (EI) [m/z (relative intensity)]: 281 (M^+, 24), 280 (16), 219 (51), 181 (100) 127 (56) 100 (23). HRMS: calcd for $C_{21}H_{15}N$: Mr = 281.1206. Found, 281.1199.

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