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Novel acyclic enediynes inhibit Cyclin A and Cdc25C expression and induce apoptosis phenomenon to show potent antitumor proliferation

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Abstract—A series of acyclic enediynes showing significant inhibition on the growth of tumor cancer is disclosed. To investigate the structure–activity relationship, compounds **12**–**33** were synthesized. Among them, compound **17** showed most potent growth inhibition activity against all tumor cell lines at low concentration, such as SR (0.4 μM) and MDA-MB-435 (0.8 μM), and almost completely blocked cell cycle in G2/M phase via controlling Cyclin A and Cdc25C expression. On the other hand, compound **29** showed potent induced apoptosis activity by inducing activation of caspase-3, -8, and -9. Thus, this article disclosed a new multiple-protein regulator in cell cycle regulation and induced apoptosis to achieve the goal of anticancer drug.

1. Introduction

Cancer cell is an abnormal cell that has been generated by complicated cell division uncontrolled and marked by continued proliferation. In the past research many small molecules can arrest cancer cell cycle machinery in G2/M phase by inhibition of Cdk family and microtubulin activity that led to the antiproliferation of cancer cell.[1] Over the past few decades, a considerable number of studies have been made on cell cycle modulators, such as Flavopiridol[2] (multiple inhibitors of Cdk family) and combretastatin A-4[3] (disrupt polymerization tubulin agent). To devise drugs that initiate apoptosis is another pathway to induce cancer into programmed cell death. So far, based on previous research we have seen that the extrinsic pathway (via death ligands) such as TRAIL or TNFα by accepting death signal and the intrinsic pathway (via mitochondrial-injury) were triggered from inside signal such as DNA damage.[4] The two apoptosis mechanisms are associated with the activation of caspase family. Both of the caspase-dependent pathways can activate caspase-3 which is the straight promoter of apoptosis. To discover natural products or synthesize small molecules that have the ability to cease cell division and initiate apoptosis is the purpose for such kind of research.

We have disclosed new novel acyclic enediyne derivatives which are different from the accustomed mode (Bergman or Myers cyclization) of enediynes which generate diradicals to destroy cells. 2-(6-Alkyl-3(*Z*)-hexen-1,5-diynyl)benzonitrile (**1**) showed remarkable cytotoxicities toward solid tumor cell in in vitro assay.[5] 2-(6-(2-Anilinyl)-3(*Z*)-hexen-1,5-diynyl)benzonitrile (**2**) represented the lowest GI_{50} values in 60 tumor cell lines, especially against the MDA-MB-435 cell of human breast cancer (0.11 μM).[6] 2-(6-(2-Thienyl)-3(*Z*)-hexen-1,5-diynyl)aniline (**3**) also showed the distinct activity for reducing cell growth and had shown time and dose-dependent antiproliferative effect on K-562 cells, but the mechanism is poorly understood.[7] Both of them can stop cell cycle machinery in G2/M phase and at the same time exhibited eminent apoptosis phenomenon. Structure–activity relationships (SAR) were also found

Keywords: Apoptosis; Enediyne; G2/M phase arrest; Cyclin A; Cdc25C.
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in C-1 and C-6 positions of acyclic enediyne core. According to our research, we found that the aryl rings bearing hetero-atoms close to C-1 and C-6 exhibited the best antiproliferation ability within enediyne derivatives. The main focus of this study is to design new group of enediynes **4**–**6** (Fig. 1) by modification of the aryl ring at C-1 and C-6, and to find out whether the aryl rings are essential to the remarkable biological activity or not. Thus, **4a**–**4d**, **5a** and **5b**, and **6a**–**6c** were synthesized.

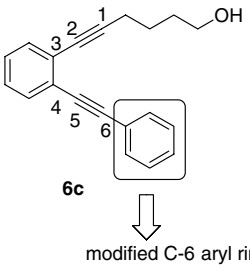
2. Results and discussion

The syntheses of compounds **4a**–**4d**, **5a** and **5b**, and **6a**–**6c** via palladium-catalyzed coupling reaction have been disclosed in the literature.[8] The growth inhibition activities of these novel enediynes were tested in the NCI's in vitro anticancer screen with the panel of 60 human tumor cell lines. Details of the test system have been published.[9] The results of compounds **4a**–**4d** and compounds **5a** and **5b** are not able to pass NCI anticancer assay. However, compounds **6a**–**6c** displayed a broad-spectrum inhibition on various cancer cell lines and the results are summarized in Table 1, especially compound **6c** shows high selectivity against SF-295 (CNS cancer) with GI_{50} of 0.15 μM.

Based on the template of **6c**, compounds **12**–**33** were synthesized for the further exploration of the SAR of this series of enediynes. The synthesis of **12**–**33** is outlined in Scheme 1. 6-(2-(2-Arylethynyl)phenyl)-5-hexyn-1-ols **12**–**33** were prepared starting from 1,2-diiodobenzene **7** and 5-hexyn-1-ol **8** via Sonogashira coupling reaction at room temperature in 62% yield. Under the same reaction conditions in room temperature, compound **10** was obtained in 84% yield by treatment of **9** with trimethylsilylacetylene. Desilylation and palladium-catalyzed coupling reaction of **10** in the presence of K_2CO_3 and MeOH with various aryl iodides gave **12**–**33** in 31–82% yields.

First of all, the in vitro result indicates that most of the average GI_{50} values of **12**–**33** ranged from 0.4 to 29.0 μM. The active compounds are shown in Table 2. Obviously, compounds **12**–**33** showed potent growth inhibition activities against various cancer cell lines including leukemia, colon cancer, CNS cancer, melanoma, ovarian cancer, prostate cancer, and breast cancer. Among them, compounds **16** (2-thieanisyl), **17** (2-trifluoromethylphenyl), and **23** (3-anisyl) showed potent activity against all tumor cell lines at lower concentration. For instance, the GI_{50} value of **17** against SR and MDA-MB-435 is 0.4 and 0.8 μM, respectively. These data indicated that the 2-position of the aryl substituent at C6 position bearing oxygen, sulfur or trifluoromethyl of the enediyne structure increased the potency of the growth inhibition activity against cancer cell lines.

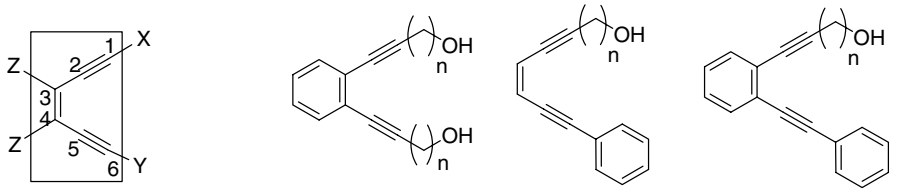
Table 1. Cytotoxic activities of **6a**–**c**



Panel/cell line	Cytotoxicity (GI_{50} in μM)[a]		
	6a	**6b**	**6c**
Leukemia/(K-562)	26.0	11.0	21.1
CNS Cancer/(SF-295)	20.6	28.1	0.15
Melanoma/(M14)	10.6	16.0	10.0
Ovarian cancer/(SK-OV-3)	16.6	17.3	10.7
Prostate cancer/(DU-145)	14.9	12.5	5.22
Breast cancer/(MDA-MB-231/ATCC)	3.26	3.41	4.29

Data obtained from the NCI's in vitro human tumor cell screen.
[a] The concentration (μM) produces 50% reduction in cell growth.

To confirm the mechanism of these synthetic enediynes **13**–**33**, we examined cell cycle phase distribution by treating K-562 cells for 24 h by flow cytometry (Table 2). Results indicated the significantly increased percentage in compounds **16**, **17**, and **23**, from 27.6% (control), to 55.7%, 86.5%, and 84.6%, respectively. The remarkable blockage of K-562 cell cycle in G2/M phase was observed on 6-(2-(2-trifluoromethylphenylethynyl))phenyl-5-hexyn-1-ol (**17**). With the exception of potent G2/M cell cycle arrest, all of the enediyne analogues were shown to induce apoptotic ability in K-562 cells. To compare the control sub-G1 area (0.96%), all compounds showed significant apoptotic activity (3.84–23.56%). More noteworthy is that compounds **25** (18.25%) and **29** (23.56%) have remarkable ability in



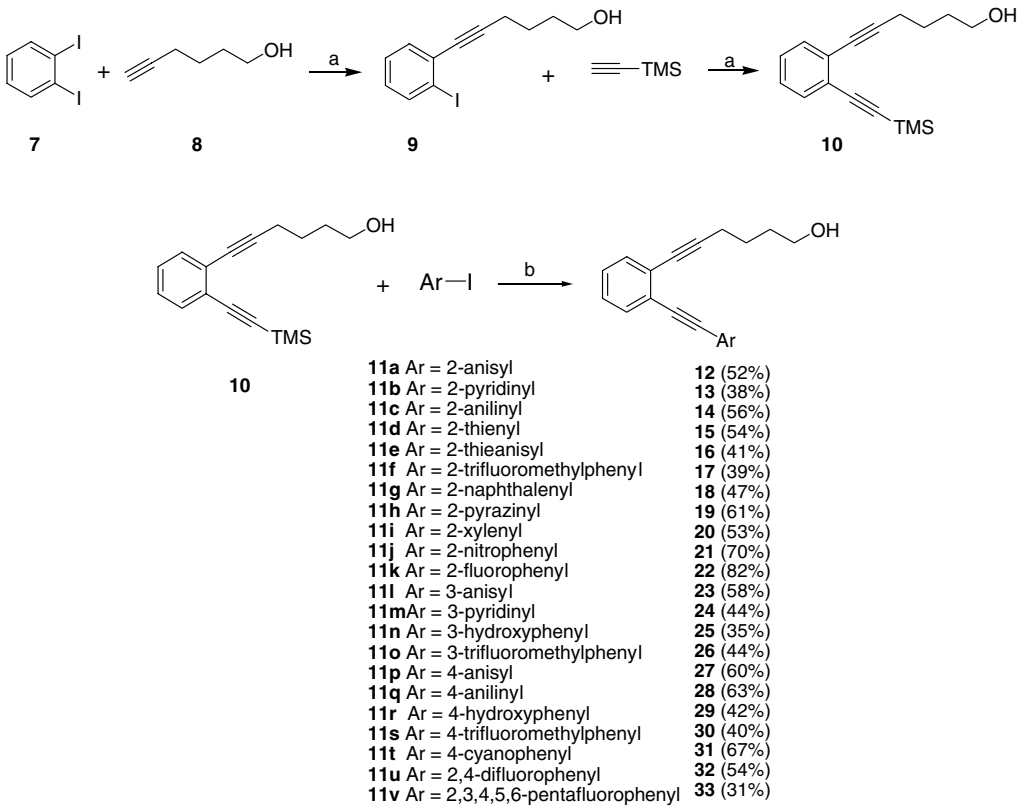
1 X= alkyl, Y= 2-cyanophenyl, Z= H
2 X= 2-anilinyl, Y= 2-cyanophenyl, Z= H
3 X= 2-anilinyl, Y= 2-thienyl, Z= H

4a n= 1
4b n= 2
4c n= 3
4d n= 4

5a n= 1
5b n= 2

6a n= 1
6b n= 2
6c n= 4

Figure 1. Chemical structures of novel enediynes **1**–**6**.



Scheme 1. Reagents and conditions: (i) $Pd(PPh_3)_4$, CuI, *n*-$BuNH_2$, ether, rt; (ii) $Pd(PPh_3)_4$, CuI, K_2CO_3, methanol, rt.

Table 2. Cytotoxicities activities and cell cycle distribution of 6-(2-(arylethynyl)phenyl)-5-hexyn-1-ol

No:	Cytotoxicity activities (GI_{50} in μM)[a]										
	12	**13**	**15**	**16**	**17**	**23**	**25**	**26**	**29**	**30**	**31**
Panel/cell line											
Leukemia/(K-562)	15.5	29.0	20.3	6.1	1.1	1.8	19.2	22.5	20.4	18.2	21.7
Leukemia/(SR)	1.0	25.5	1.8	3.5	0.4	1.9	NA	18.3	NA	0.6	NA
Colon cancer/(HCT-15)	19.9	29.8	13.1	3.8	3.5	3.6	13.6	16.3	14.1	12.5	15.6
CNS cancer/(SF-295)	22.1	21.8	19.0	1.5	2.3	7.9	21.0	16.5	16.2	19.1	21.5
Melanoma/(LOX IMVI)	17.9	27.7	13.9	5.8	4.9	6.3	16.7	17.5	13.6	17.1	18.3
Ovarian cancer/(OVCAR-3)	19.8	26.1	15.0	14.7	2.3	10.7	20.0	16.8	14.8	17.8	19.9
Prostate cancer/(DU-145)	14.3	9.8	13.0	4.7	6.1	4.1	17.4	15.3	16.8	15.8	16.0
Breast cancer/(MDA-MB-435)	20.2	25.1	16.6	3.9	0.8	1.9	16.1	16.9	16.1	16.3	18.9
Cell cycle percentage[b]											
G0/G1 (%)	30.9	30.4	22.7	19.8	2.7	3.4	26.7	30.6	25.6	36.9	51.2
S (%)	41.5	52.6	47.5	24.6	10.8	11.9	37.1	40.0	39.5	38.3	38.0
G2/M (%)	27.6	17.0	29.8	55.7	86.5	84.6	36.2	29.4	34.9	24.8	10.8
Sub-G1 (apoptosis area)	8.08	4.40	5.23	9.23	10.47	10.81	18.25	3.84	23.56	5.36	5.65

Data obtained from the NCI's in vitro human tumor cell screen. NA=Not available.

[a] The concentrations (μM) corresponding to 50% growth inhibition.

[b] The percentages of the cells in each phase were calculated by using the WinMDI software for the flow cytometry treated with sample for 24 h used at a concentration of 50 μM. Control percentage: G0/G1 (32.3%), S (48.4%), G2/M (19.3%), and sub-G1 (0.96%).

inducing apoptosis as compared to control. These results suggested that novel enediyne derivatives induced G2/M arrest and apoptosis in K-562 cells.

To obtain the understanding of mechanism underlying G2/M arrest in these enediyne analogues, we examined the expression of G2/M associated proteins as shown in Figure 2b, including Cyclin A, B, Cdk1, 2, and Cdc25C. According to Figure 2b, we found the protein levels of Cyclin A and Cdc25C were decreased by the treatment of compound **17** in 25 and 50 μM. In contrast, the protein level of Cyclin B, Cdk1, and Cdk2 did not change. In G2 phase, Cyclin B/Cdk1 is at an inactive form by phosphorylation of Thr 14 and Tyr 15, which are dephosphorylated by Cdc25C before entering into mitosis.[10] Compound **17** could inhibit the expression

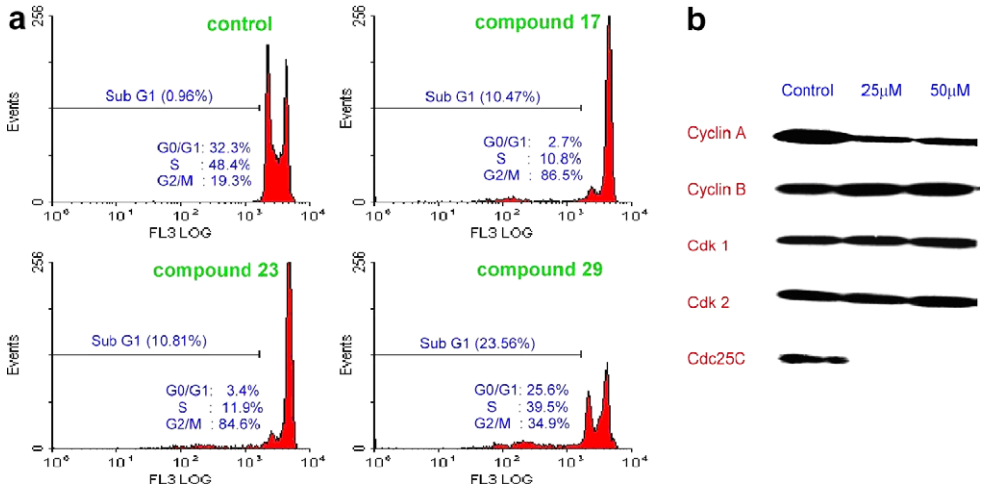


Figure 2. (a) Cell cycle analysis. Leukemia (K-562 cells) was cultured with 50 μM of compounds **17**, **23**, and **29** for 24 h and analyzed by flow cytometry. G0/G1, S, and G2/M indicated the phase and sub-G1 area refers to the portion of apoptotic cells. (b) Expression of the G2/M-associated proteins. K-562 cells were treated with 25 and 50 μM of compound **17**. Immunoblot analysis for the levels of cell cycle regulatory proteins, including Cyclin A, B, Cdk1, Cdk2, and Cdc25C.

of protein Cdc25C and Cyclin A, and then cease cell cycle machinery in G2/M phase.

It is known that the induction of caspases plays a major role in the apoptosis-signaling pathway. We determined the kinetics of caspase-3, -8, and -9 activation by compound **29** as shown in Figure 3a. Caspase-3 activity (1.4- and 2.6-fold of control), caspase-8 activity (1.2- and 1.8-fold of control), and caspase-9 activity (1.5- and 1.7-fold of control) were increased at 24 h of treatment with compound **29** (25 and 50 μM). In order to confirm the role of caspases in compound **29**-induced apoptosis, we pretreated K-562 cells with membrane-permeable caspase-3-specific inhibitor (Z-DEVD-FMK), caspase-8-specific inhibitor (Z-IETD-FMK), and caspase-9-specific inhibitor (Z-LEHD-FMK), and then were followed by treatment with 50 μM of compound **29**. It demonstrated clearly compound **29**-induced apoptosis is significantly reduced from 44% (lane a) to 17% (lane b), 19% (lane c), and 42% (lane d). These results indicated that novel enediyne-induced apoptosis is associated with activities of caspase-3, -8, and -9. Large percentage decreased when caspase-3 (line b) and caspase-8 (line c) inhibitors are added, which then proved compound **29** as a trigger of apoptosis via the extrinsic pathway.

3. Conclusion

From the structure–activity relationship (shown in Fig. 4) study of compounds **12**–**33**, we obtain biological activity when the following structures are present: (i) the C1 position of enediyne core needed alkanyl alcohol ($n = 4$), (ii) the phenyl substituent of the C3–C4 position in enediyne core, (iii) the 2-substituted (O, S, and CF_3) aryl in C6 position of enediyne, especially compound **17** (where the C6 aryl group is 2-trifluoromethylphenyl) shows highest potency of growth inhibition activity against SR (0.4 μM) and MDA-MB-435 (0.8 μM) cell lines. Based on cytotoxicity, cell cycle assay, immunoblot analysis, and caspase-3, -8, and -9 colorimetric assay, we found out an all new class of acyclic enediyne derivatives that are potent in growth inhibition against all 60 human tumor cancer cells. Compound **17** also shows potent G2/M arrest by inhibiting Cyclin A and Cdc25C protein expressions and compound **29** induces apoptosis by inducing activation of caspase-3, -8, and -9. In this article, we highlight a novel enediyne antitumor proliferation mechanism; this new research will be helpful in further elucidation of undiscovered biological properties of these novel antitumor enediynes.

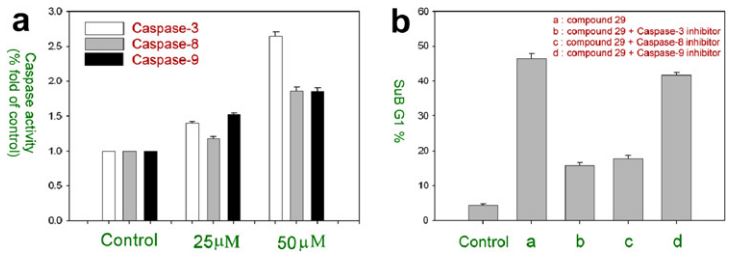


Figure 3. Effects of compound **29** on caspase-3, -8, and -9 protein expression. (a) The caspase-3, -8, and -9 colorimetric assay in 25 and 50 μM for 24 h. (b) The sub-G1 area calculated by flow cytometry. Cells were pre-incubated with or without the caspase inhibitor. Z-DEVD-FMK (cas-3 inhibitor), Z-IETD-FMK (cas-8 inhibitor), and Z-LEHD-FMK (cas-9 inhibitor) for 24 h and this was followed by treatment with compound **29** in 50 μM.

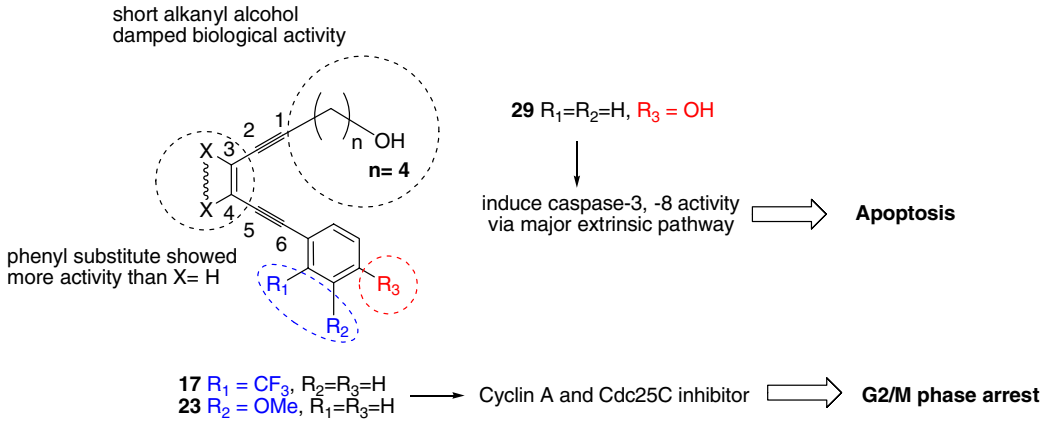


Figure 4. The structure–activity relationship of novel acyclic enediyne.

4. Experimental

4.1. General procedure for coupling compounds 12–33

To a degassed solution of compound **10** (12 mmol) containing CuI (3.2 mmol) and K_2CO_3 (30 mmol) in MeOH (15 ml) was added a degassed solution of aryl iodides (**11a**–**11v**) (12 mmol) containing $Pd(PPh_3)_4$ (0.8 mmol) in MeOH (20 ml). The resulting reaction mixture was stirred for 6 h and after removal of methanol in vacuo, 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.

4.2. Cell culture

Human leukemia K-562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and human purified lymphocyte preparation was obtained from blood as described previously. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μM glutamine, and antibiotics (100 U/ml penicillin and 100 μ/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

4.3. Cell cycle analysis

Flow cytometry was used to measure cell cycle profile and apoptosis. For cell cycle analysis, K-562 cells treated with compounds **12**–**33** (50 μM) for 24 h were harvested by centrifugation. After being washed with PBS, the cells were fixed with ice-cold 70% ethanol for 30 min, washed with PBS, and then treated with 1 ml of 1 mg/ml of RNase A solution at 37 °C for 30 min. Cells were harvested by centrifugation at 1000 rpm for 5 min and further stained with 250 μl of DNA staining solution (10 mg of propidium iodide [PI], 0.1 mg of trisodium citrate, and 0.03 ml of Triton X-100 were dissolved in 100 ml H_2O) at room temperature for 30 min in the dark. After loading 500 μl of PBS, the DNA contents of 10,000 events were measured by FACScan (Elite ESP, Beckman Coulter, Brea, CA) and the cell cycle profile was analyzed from the DNA content histograms with WinCycle software. When cells were undergoing apoptosis the contained DNA was digested by endonuclease then the sub-G1 peak appeared. The percentage in sub-G1 was analyzed by gating on cell cycle dot blots using Windows Multiple Document Interface software (WinMDI).

4.4. Western-blotting analysis

Cells were washed in PBS, suspended in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and placed on ice for 30 min. After centrifugation at 20,000*g* for 30 min at 4 °C, the supernatant was collected. The protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Whole lysate (50 μg) was resolved by 12% SDS–PAGE, transferred onto PVDF membranes (Roche) by electroblotting, and probed with anti-p21, -p27, anti-Cdk1, -Cdk2, anti-Cyclin A, -CyclinB1, and anti-Cdc25C (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blot was developed by enhanced chemiluminescence.

4.5. Caspase colorimetric assay

After different treatments, cells (10^6 cells/ml) were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 18,000*g* for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colorigenic substrates at 37 °C for 1 h. The activity of caspases was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

4.6. Caspase inhibitor assay

Cells were pre-incubated with or without the caspase inhibitors (caspase-3: Z-DEVD-FMK; caspase-8: ZIETD-FMK; caspase-9: Z-LEHD-FMK) for 1 h and this was followed by treatment with or without 50 μM of the compound **29** for 24 h. Control and treated cells

were harvested, washed in cold PBS, and fixed in 70% ethanol. DNA was treated with RNase A solution (500 U/ml) at 37 °C for 15 min and stained by propidium iodide (50 μg/ml) in PBS. DNA contents were analyzed by the flow cytometric analysis with the WinMDI software.

4.7. 6-(2-Iodophenyl)hex-5-yn-1-ol (9)

To a degassed solution of compound **8** (12 mmol) containing CuI (3.2 mmol) and *n*-$BuNH_2$ (30 mmol) in ether (15 ml) was added a degassed solution of 1,2-diiodobenzene **7** (12 mmol) containing $Pd(PPh_3)_4$ (0.8 mmol) in ether (20 ml). The resulting reaction mixture was stirred for 4 h and then 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 that were obtained in 62% yield as yellowish oil. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.81 (d, 1H, J = 7.6 Hz), 7.39 (d, 1H, J = 7.6 Hz), 7.26 (td, 1H, J = 6.4, 0.4 Hz), 6.95 (td, 1H, J = 7.6, 1.6 Hz), 3.72 (t, 2H, J = 6.4 Hz), 2.52 (t, 2H, J = 6.8 Hz), 1.83–1.72 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.5, 132.4, 130.3, 128.7, 127.7, 101.0, 94.2, 83.2, 62.4, 31.9, 24.7, 19.3. MS (EI) [*m*/*z* (relative intensity)] 300 (M^+, 5), 173 (100). HRMS Calcd for $C_{12}H_{13}OI$, Mr = 300.0011; found: 300.0016.

4.8. 6-(2-(2-Trimethylsilylethynyl))phenylhex-5-yn-1-ol (10)

To a degassed solution of trimethylsilylacetylene (12 mmol) containing CuI (3.2 mmol) and *n*-$BuNH_2$ (30 mmol) in ether (15 ml) was added a degassed solution of compound **9** (12 mmol) containing $Pd(PPh_3)_4$ (0.8 mmol) in ether (20 ml). The resulting reaction mixture was stirred for 4 h and then 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 that were obtained in 84% yield as brown oil. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.44 (d, 1H, J = 6.8 Hz), 7.37 (d, 1H, J = 6.8 Hz), 7.26–7.17 (m, 2H), 3.71 (t, 2H, J = 6.4 Hz), 2.51 (t, 2H, J = 6.8 Hz), 1.77–1.69 (m, 4H), 0.26 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 132.2, 131.8, 128.1, 127.2, 126.7, 125.4, 103.8, 97.8, 94.2, 79.6, 62.4, 31.9, 25.0, 19.4, 0.1. MS (EI) [*m*/*z* (relative intensity)] 270 (M^+, 85), 255 (58), 209 (46), 165 (100). HRMS Calcd for $C_{17}H_{22}OSi$, Mr = 270.1440; found: 270.1434.

4.9. 6-(2-(2-Anisylethynyl))phenyl-5-hexyn-1-ol (12)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 52% of yellow oil according to general procedure. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.55–7.50 (m, 2H), 7.43–7.40 (m, 1H), 7.34–7.22 (m, 3H), 6.97–6.90 (m, 2H), 3.91 (s, 3H), 3.58 (t, 2H, J = 6.4 Hz), 2.53 (t, 2H, J = 6.4 Hz), 1.76–1.57 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.9, 133.7, 131.9, 131.7, 131.5, 129.8, 127.8, 127.2, 126.2, 125.9, 120.5, 112.6, 110.9, 94.2, 89.1, 79.9, 62.4, 55.9, 31.8, 24.9, 19.4. MS (EI) [*m*/*z* (relative intensity)] 304 (M^+, 100), 281 (53), 215 (69), 202 (62). HRMS Calcd for $C_{21}H_{20}O_2$, Mr = 304.1463; found: 304.1472.

4.10. 6-(2-(2-Pyridinylethynyl))phenyl-5-hexyn-1-ol (13)

The compound was purified by column chromatography, eluting with hexane/EA (1:1), to give 38% of brown oil according to general procedure. ^1H NMR ($CDCl_3$, 400 MHz) δ 8.56 (d, 1H, J = 4.8 Hz), 7.68 (td, 1H, J = 7.6, 2.0 Hz), 7.56–7.53 (m, 2H), 7.41 (d, 1H, J = 7.2 Hz), 7.29–7.21 (m, 3H), 3.64 (t, 2H, J = 6.4 Hz), δ 3.01 (br s, 2H), 2.52 (t, 2H, J = 6.4 Hz), 1.73–1.54 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 149.6, 143.0, 136.5, 132.5, 131.9, 128.7, 127.8, 127.2, 126.6, 124.3, 123.2, 95.0, 91.2, 89.0, 79.5, 62.0, 31.9, 24.8, 19.4. MS (EI) [*m*/*z* (relative intensity)] 275 (M^+, 5), 230 (100). HRMS Calcd for $C_{19}H_{17}ON$, Mr = 275.1310; found: 275.1297.

4.11. 6-(2-(2-Anilinylethynyl))phenyl-5-hexyn-1-ol (14)

The compound was purified by column chromatography, eluting with hexane/EA (2:1), to give 56% of brown oil according to general procedure. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.51 (d, 1H, J = 7.2 Hz), 7.44 (d, 1H, J = 6.4 Hz), 7.38 (d, 1H, J = 7.6 Hz), 7.36–7.24 (m, 2H), 7.15 (t, 2H, J = 6.4 Hz), 6.78–6.71 (m, 2H), 3.62 (t, 2H, J = 6.0 Hz), 2.52 (t, 2H, J = 6.8 Hz), 1.74–1.68 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.5, 132.0, 131.9, 131.4, 129.9, 127.8, 127.5, 125.8, 125.5, 118.1, 114.5, 108.1, 94.1, 93.9, 89.4, 80.3, 62.4, 31.8, 25.0, 19.5. MS (EI) [*m*/*z* (relative intensity)] 289 (M^+, 51), 231 (86), 230 (100). HRMS Calcd for $C_{20}H_{19}ON$, Mr = 289.1467; found: 289.1460.

4.12. 6-(2-(2-Thienylethynyl))phenyl-5-hexyn-1-ol (15)

The compound was purified by column chromatography, eluting with hexane/EA (2:1), to give 54% of brown oil according to general procedure. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.50–7.47 (m, 1H), 7.43–7.41 (m, 1H), 7.32–7.29 (m, 2H), 7.27–7.24 (m, 2H), 7.04–7.01 (m, 1H), 3.64 (t, 2H, J = 6.4 Hz), 2.55 (t, 2H, J = 6.4 Hz), 1.80–1.70 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 131.9, 131.7, 131.5, 128.1, 127.5, 127.3, 127.1, 126.2, 125.3, 123.4, 94.6, 92.2, 85.9, 79.6, 62.4, 31.9, 24.9, 19.4. MS (EI) [*m*/*z* (relative intensity)] 280 (M^+, 100), 221 (53). HRMS Calcd for $C_{18}H_{16}OS$, Mr = 280.0922; found: 280.0912.

4.13. 6-(2-(2-Thieanisylethynyl))phenyl-5-hexyn-1-ol (16)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 41% of yellowish oil according to general procedure. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.57–7.55 (m, 1H), 7.51 (d, 1H,

J = 7.6 Hz), 7.44–7.42 (m, 1H), 7.34–7.29 (m, 1H), 7.28–7.24 (m, 2H), 7.19 (d, 1H, J = 7.6 Hz), 7.12 (td, 1H, J = 7.2, 0.8 Hz), 3.59 (t, 2H, J = 6.4 Hz), 2.54 (t, 2H, J = 6.4 Hz), 1.76–1.68 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 141.6, 132.5, 132.0, 131.9, 128.8, 128.1, 127.3, 126.2, 125.5, 124.2, 124.1, 121.4, 94.8, 94.5, 90.0, 79.8, 62.5, 31.9, 24.9, 19.6, 15.1. MS (EI) [m/z (relative intensity)] 320 (M^+, 16), 247 (100). HRMS Calcd for $C_{21}H_{20}OS$, Mr = 320.1235; found: 320.1224.

4.14. 6-(2-(2-Trifluoromethylphenylethynyl))phenyl-5-hexyn-1-ol (17)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 39% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69 (d, 2H, J = 8.4 Hz), 7.54–7.51 (m, 2H), 7.45–7.40 (m, 2H), 7.30–7.24 (m, 2H), 3.64 (t, 2H, J = 6.4 Hz), 2.54 (t, 2H, J = 6.4 Hz), 1.78–1.69 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 134.1, 132.3, 132.1, 131.3, 128.8, 128.5, 128.0, 127.3,, 125.9, 125.8, 122.2, 121.6, 119.5, 94.6, 93.9, 88.4, 79.4, 62.4, 31.9, 24.9, 19.4. MS (EI) [m/z (relative intensity)] 342 (M^+, 77), 283 (83), 271 (84), 262 (100), 233 (70). HRMS Calcd for $C_{21}H_{17}OF_3$, Mr = 342.1232; found: 342.1238.

4.15. 6-(2-(2-Naphthalenylethynyl))phenyl-5-hexyn-1-ol (18)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 47% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.62 (d, 1H, J = 8.0 Hz), 7.87 (t, 2H, J = 7.2 Hz), 7.79 (d, 1H, J = 7.2 Hz), 7.63–7.46 (m, 5H), 7.31–7.28 (m, 2H), 3.50 (t, 2H, J = 6.0 Hz), 2.58 (t, 2H, J = 6.8 Hz), 1.71–1.67 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 133.3, 133.2, 132.0, 131.9, 130.4, 128.8, 128.3, 128.0, 127.4, 126.7, 126.5, 126.4, 126.4, 125.7, 125.3, 121.0, 94.5, 93.4, 90.9, 80.1, 62.4, 31.9, 25.0, 19.6. MS (EI) [m/z (relative intensity)] 324 (M^+, 61), 167 (61), 165 (100). HRMS Calcd for $C_{24}H_{20}O$, Mr = 324.1514; found: 324.1512.

4.16. 6-(2-(2-Pyrazinylethynyl)phenyl)-5-hexyn-1-ol (19)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 61% of yellow oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.80 (d, 1H, J = 1.2 Hz), 8.57 (d, 1H, J = 2.4 Hz), 8.50 (d, 1H, J = 2.8 Hz), 7.60 (dd, 1H, J = 7.6, 1.6 Hz), 7.45 (d, 1H, J = 7.6 Hz), 7.36–7.26 (m, 2H), δ 3.68 (t, 2H, J = 6.4 Hz), 2.57 (t, 2H, J = 6.4 Hz), 1.85–1.81 (m, 2H), 1.80–1.72 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.2, 144.3, 142.8, 140.3, 132.6, 132.1, 129.3, 127.4, 127.1, 123.7, 95.3, 92.8, 88.6, 79.4, 62.4, 31.9, 24.9, 19.5. MS (EI) [m/z (relative intensity)] 276 (M^+, 11), 231 (100), 219 (99). HRMS Calcd for $C_{18}H_{16}ON_2$, Mr = 276.1263; found: 276.1245.

4.17. 6-(2-(2-Xylenylethynyl)phenyl)-5-hexyn-1-ol (20)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 53% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.53–7.51 (m, 1H), 7.45–7.42 (m, 1H), 7.41–7.39 (m, 1H), 7.27–7.23 (m, 2H), 7.14–7.07 (m, 2H), 3.61 (t, 2H, J = 6.4 Hz), 2.53 (t, 2H, J = 6.4 Hz), 2.52 (s, 3H), 2.31 (s, 3H), 1.76–1.69 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.6, 136.8, 132.0, 131.8, 130.0, 129.9, 127.7, 127.3, 126.1, 125.9, 125.3, 123.2, 94.1, 92.4, 91.7, 80.0, 62.4, 31.9, 24.9, 20.3, 19.6, 17.5. MS (EI) [m/z (relative intensity)] 302 (M^+, 100). HRMS Calcd for $C_{22}H_{22}O$, Mr = 302.1671; found: 302.1670.

4.18. 6-(2-(2-Nitrophenylethynyl))phenyl-5-hexyn-1-ol (21)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 70% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.07 (dd, 1H, J = 7.6, 1.6 Hz), 7.74 (dd, 1H, J = 7.6, 1.6 Hz), 7.61 (dd, 1H, J = 7.6, 1.6 Hz), 7.58–7.56 (m, 1H), 7.48–7.43 (m, 2H), 7.32–7.25 (m, 2H), 3.66 (t, 2H, J = 6.4 Hz), 2.57 (t, 2H, J = 6.4 Hz), 1.77–1.70 (m, 4H), 1.59 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 134.6, 134.6, 132.5, 132.3, 131.9, 128.6, 128.4, 127.1, 126.5, 124.4, 124.3, 118.6, 95.9, 94.8, 87.5, 79.0, 62.1, 31.6, 24.7, 19.2. MS (EI) [m/z (relative intensity)] 319 (M^+, 31), 232 (78), 219 (100). HRMS Calcd for $C_{20}H_{17}O_3N$, Mr = 319.1208; found: 319.1210.

4.19. 6-(2-(2-Fluorophenylethynyl))phenyl-5-hexyn-1-ol (22)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 82% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.56–7.52 (m, 2H), 7.43 (t, 1H, J = 6.8 Hz), 7.34–7.25 (m, 3H), 7.15–7.08 (m, 2H), 3.63 (t, 2H, J = 6.0 Hz), 2.55 (t, 2H, J = 6.4 Hz), 1.78–1.69 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.8, 161.3, 133.5, 131.8, 130.1, 128.3, 127.3, 126.5, 125.2, 124.0, 115.6, 115.4, 94.7, 93.5, 86.0, 79.5, 62.4, 31.9, 24.9, 19.4. MS (EI) [m/z (relative intensity)] 292 (M^+, 39), 233 (100). HRMS Calcd for $C_{20}H_{17}OF$, Mr = 292.1263; found: 292.1260.

4.20. 6-(2-(3-Anisylethynyl))phenyl-5-hexyn-1-ol (23)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 58% of yellow oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51 (d, 1H, J = 6.0 Hz), 7.42 (d, 1H, J = 6.0 Hz), 7.29–7.24 (m, 3H), 7.15 (d, 1H, J = 7.6 Hz), 7.09 (s, 1H), 6.91 (d, 1H, J = 7.2 Hz), 3.82 (s, 3H), 3.61 (t, 2H, J = 6.4 Hz), 2.54 (t, 2H, J = 6.8 Hz), 1.77–1.72 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.3, 131.8, 131.8, 129.4, 128.0, 127.3, 126.4, 125.5, 124.4, 124.2, 116.7, 114.7, 94.4, 92.6, 88.3, 79.8, 62.4, 55.3, 31.9, 25.0, 19.5. MS (EI) [m/z (relative intensity)] 304 (M^+, 100), 215 (57). HRMS Calcd for $C_{21}H_{20}O_2$, Mr = 304.1463; found: 304.1472.

4.21. 6-2-(3-Pyridinylethynyl)phenyl-5-hexyn-1-ol (24)

The compound was purified by column chromatography, eluting with hexane/EA (2:1), to give 44% of brown

oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.79 (s, 1H), 8.53 (d, 1H, J = 4.8 Hz), 7.83 (dt, 1H, J = 8.0, 1.6 Hz), 7.52–7.43 (m, 2H), 7.32–7.25 (m, 3H), 3.63 (t, 2H, J = 6.4 Hz), 2.54 (t, 2H, J = 6.0 Hz), 1.77–1.70 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 152.1, 148.1, 138.6, 132.0, 131.8, 128.5, 127.4, 126.7, 124.7, 123.2, 120.8, 94.8, 92.0, 89.0, 79.5, 62.1, 31.8, 25.2, 19.4. MS (EI) [*m*/*z* (relative intensity)] 275 (M^+, 89), 230 (100). HRMS Calcd for $C_{19}H_{17}ON$, *Mr* = 275.1310; found: 275.1317.

4.22. 6-(2-(3-Hydroxyphenylethynyl))phenyl-5-hexyn-1-ol (25)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 35% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51 (d, 1H, J = 6.4 Hz), 7.42 (d, 1H, J = 6.8 Hz), 7.26–7.21 (m, 3H), 7.19 (s, 1H), 7.08 (d, 1H, J = 7.6 Hz), 6.85 (d, 1H, J = 6.8 Hz), 3.79 (t, 2H, J = 6.0 Hz), 2.55 (t, 2H, J = 6.8 Hz), 1.85–1.82 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.3, 131.8, 131.8, 129.7, 127.9, 127.4, 126.2, 125.6, 124.0, 123.4, 118.3, 116.3, 94.1, 92.8, 88.2, 79.9, 63.1, 32.0, 25.8, 19.7. MS (EI) [*m*/*z* (relative intensity)] 290 (M^+, 97), 215 (61), 202 (100). HRMS Calcd for $C_{20}H_{18}O_2$, *Mr* = 290.1307; found: 290.1298.

4.23. 6-(2-(3-Trifluoromethylphenylethynyl))phenyl-5-hexyn-1-ol (26)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 44% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.81 (s, 1H), δ 7.71 (d, 1H, J = 7.6 Hz), δ 7.58 (d, 1H, J = 7.6 Hz), δ 7.52–7.43 (m, 2H), δ 7.30–7.26 (m, 2H), δ 3.63 (t, 2H, J = 6.0 Hz), δ 2.55 (t, 2H, J = 6.4 Hz), δ 1.77–1.72 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 134.6, 132.0, 131.8, 128.9, 128.5, 128.5, 128.4, 127.5, 126.6, 124.9, 124.8, 124.8, 124.3, 94.6, 91.1, 90.1, 79.6, 62.3, 31.8, 25.0, 19.4. MS (EI) [*m*/*z* (relative intensity)] 342 (M^+, 93), 283 (93), 213 (88), 169 (100). HRMS Calcd for $C_{21}H_{17}OF_3$, *Mr* = 342.1232; found: 342.1214.

4.24. 6-(2-(4-Anisylethynyl))phenyl-5-hexyn-1-ol (27)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 60% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51–7.48 (m, 3H), 7.43–7.40 (m, 1H), 7.26–7.22 (m, 2H), 6.88 (d, 2H, J = 6.8 Hz), 3.83 (s, 3H), 3.62 (t, 2H, J = 6.0 Hz), 2.55 (t, 2H, J = 6.4 Hz), 1.78–1.69 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.7, 133.1, 133.1, 131.8, 131.6, 127.6, 127.3, 126.1, 125.9, 115.4, 114.0, 114.0, 94.2, 92.9, 87.2, 79.9, 62.4, 55.3, 31.9, 25.0, 19.5. MS (EI) [*m*/*z* (relative intensity)] 304 (M^+, 100), 202 (72). HRMS Calcd for $C_{21}H_{20}O_2$, *Mr* = 304.1463; found: 304.1458.

4.25. 6-(2-(4-Anilinylethynyl))phenyl-5-hexyn-1-ol (28)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 60% of yellow oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51–7.46 (m, 4H), 6.86–6.83 (m, 2H), 6.21 (d, 2H, J = 8.8 Hz), 3.31 (t, 2H, J = 6.0 Hz), 2.31 (t, 2H, J = 6.4 Hz), 1.60–1.51 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.2, 134.1, 133.9, 132.8, 132.6, 131.5, 129.8, 128.2, 127.6, 125.4, 115.4, 113.4, 96.7, 95.4, 88.0, 80.6, 62.9, 32.9, 26.1, 20.4. MS (EI) [*m*/*z* (relative intensity)] 289 (M^+, 32), 167 (100). HRMS Calcd for $C_{20}H_{19}ON$, *Mr* = 289.1467; found: 289.1454.

4.26. 6-(2-(4-Hydroxyphenylethynyl))phenyl-5-hexyn-1-ol (29)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 42% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.49–7.40 (m, 4H), 7.24–7.22 (m, 2H), 6.83–6.79 (m, 2H), 3.62 (t, 2H, J = 6.0 Hz), 2.54 (t, 2H, J = 6.8 Hz), 1.79–1.66 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.1, 133.3, 133.3, 131.8, 131.7, 127.7, 127.3, 126.1, 125.9, 115.6, 115.6, 115.5, 94.1, 92.7, 87.1, 80.0, 62.5, 31.8, 24.9, 19.4. MS (EI) [*m*/*z* (relative intensity)] 290 (M^+, 100), 202 (63). HRMS Calcd for $C_{20}H_{18}O_2$, *Mr* = 290.1307; found: 290.1299.

4.27. 6-(2-(4-Trifluoromethylphenylethynyl))phenyl-5-hexyn-1-ol (30)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 40% of yellow oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66–7.59 (m, 4H), 7.52 (d, 1H, J = 6.4 Hz), 7.44 (d, 1H, J = 6.4 Hz), 7.30–7.25 (m, 2H), 3.64 (t, 2H, J = 6.4 Hz), 2.55 (t, 2H, J = 6.8 Hz), 1.78–1.71 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ132.0, 131.9, 131.8, 129.7, 128.5, 127.4, 127.2, 126.6, 125.3, 125.3, 125.2, 125.2, 124.8, 94.6, 91.2, 90.8, 79.6, 62.3, 31.8, 25.0, 19.4. MS (EI) [*m*/*z* (relative intensity)] 342 (M^+, 96), 296 (77), 283 (77), 213 (100). HRMS Calcd for $C_{21}H_{17}OF_3$, *Mr* = 342.1232; found: 342.1220.

4.28. 6-(2-(4-Cyanophenylethynyl))phenyl-5-hexyn-1-ol (31)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 67% of yellow oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66–7.60 (m, 4H), 7.51 (d, 1H, J = 6.4 Hz), 7.45 (d, 1H, J = 6.4 Hz), 7.32–7.25 (m, 2H), 3.65 (t, 2H, J = 6.4 Hz), 2.55 (t, 2H, J = 6.8 Hz), 1.78–1.71 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 132.1, 132.1, 132.0, 132.0, 128.8, 128.8, 128.3, 127.4, 127.4, 126.7, 124.4, 118.5, 111.5, 94.8, 92.9, 90.9, 79.5, 62.3, 31.8, 25.0, 19.4. MS (EI) [*m*/*z* (relative intensity)] 299 (M^+, 67), 254 (67), 240 (100). HRMS Calcd for $C_{21}H_{17}ON$, *Mr* = 299.1310; found: 299.1303.

4.29. 6-(2-(2,4-Difluorophenylethynyl))phenyl-5-hexyn-1-ol (32)

The compound was purified by column chromatography, eluting with hexane/EA (3:1), to give 54% of brown

oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.54–7.49 (m, 2H), 7.44–7.42 (m, 1H), 7.29–7.23 (m, 2H), 6.91–6.85 (m, 2H), 3.65 (t, 2H, J = 6.0 Hz), 2.54 (t, 2H, J = 6.4 Hz), 1.78–1.70 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 163.9, 161.4, 134.3, 134.2, 131.9, 131.9, 128.3, 127.3, 126.4, 125.0, 111.7, 104.3, 94.7, 93.2, 84.9, 79.5, 62.4, 31.8, 24.9, 19.4. MS (EI) [*m*/*z* (relative intensity)] 310 (M^+, 65), 264 (52), 251 (100). HRMS Calcd for $C_{20}H_{16}OF_2$, Mr = 310.1169; found: 310.1163.

4.30. 6-(2-(2,3,4,5,6-Pentafluorophenylethynyl))phenyl-5-hexyn-1-ol (33)

The compound was purified by column chromatography, eluting with hexane/EA (4:1), to give 31% of brown oil according to general procedure. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.55 (d, 1H, J = 7.2 Hz), 7.45 (d, 1H, J = 7.6 Hz), 7.35–7.30 (m, 2H), 3.68 (t, 2H, J = 6.0 Hz), 2.54 (t, 2H, J = 6.8 Hz), 1.77–1.70 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 132.2, 132.2, 132.0, 132.0, 132.0, 129.3, 129.3, 129.0, 127.4, 127.4, 126.8, 123.9, 100.6, 95.4, 78.9, 75.0, 62.4, 31.9, 24.8, 19.3. MS (EI) [*m*/*z* (relative intensity)] 364 (M^+, 48), 318 (80), 287 (100). HRMS Calcd for $C_{20}H_{13}OF_5$, Mr = 364.0880; found: 364.0883.

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