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Synthesis and in vitro anti-proliferative activity of β-elemene monosubstituted derivatives in HeLa cells mediated through arrest of cell cycle at the G1 phase

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ABSTRACT

β-Elemene monosubstituted amine, ether and rhenium coordinated complex were synthesized. Their structures were characterized by IR, ^{1}H NMR, ^{13}C NMR, HRMS or EA. Their IC_{50} on HeLa cell lines, cell cycle and protein expression of G_1 phase (Cyclin D_1, Rb, P-Rb) were detected respectively by the method of WST-1, Flow Cytometry and Western Blot. The Results showed that the in vitro anti-proliferative activity of β-elemene monosubstituted amine and $Re(CO)_3$-β-elemene derivatives in human cervix epitheloid carcinoma HeLa cells were improved significantly compared with both of ether derivatives and parent β-elemene. These derivatives could reduce Rb phosphorylation and cyclin D_1 protein expression to arrest the cell cycle at G_1 phase.

1. Introduction

β-Elemene, (5*S*,7*R*,10*S*)-(−)-(1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane), a natural sesquiterpene extracted from the Traditional Chinese Herb Medicinal Curcuma *wenyujin*,[1] is the main effective monomer of elemene emulsion. β-Elemene exhibits anticancer effects in human and murine tumour cells in vitro and in vivo and has substantial clinical activity against various tumours without severe side effects.[2–4] No bone marrow suppression and drug resistance have been observed in the clinical studies; on the contrary, patient immunity was improved during the therapy with β-elemene.[5,6] In China β-elemene has been effective for the treatment of cervical cancer as well as carcinomas of the liver, brain and other vital tissues, and now application for clinical studies in the US. However β-elemene suffers from limited bioavailability due to poor water solubility, short half lives and rapid clearance from the body. The mechanism of action of β-elemene in cancer remains unknown (Fig. 1).

Some of the β-elemene derivatives with better properties have already been developed.[7,8] As we know the mixture of β-elemene 13-monosubstituted, 14-monosubstituted and 13,14-disubstituted derivatives is difficult to separate each other, so an easy way to solve the problem is to let β-elemene react with much excess another reactant to give 13,14-disubstituted derivatives. Dong reported the β-elemene 13,14-disubstituted amine derivatives' activity in cancer cells improved significantly.[7] Peng et al.[6] summarized the methodological quality of these trials for β-elemene derivatives' synthesis is relatively poor. We for the first time to yield the pure 13-monosubstituted ether, amine and rhenium complex derivatives by carefully controlling the reaction conditions, to our pleasure some of the derivatives has much higher anti-proliferative activities compared with that of parent β-elemene, furthermore we detected the cell growth and cell cycle of some derivatives and explored their possible mechanism of their anti-proliferative activities. Herein we report our preliminary result.

2. Results and discussion

2.1. Chemical syntheses

The monosubstituted β-elemene ether and amine derivatives were synthesized according to the procedure outlined in Scheme 1. The synthesis began with chlorination of β-elemene with NaClO, the resulting product chlorinated β-elemene **2** reacted further with alcohols or amine to yield the desired β-elemene monosubstituted ether or amine derivatives **3, 4 (a–h)**. Generally speaking the first step product is mixture of 13-monosubstituted, 14-monosubstituted, disubstituted chlorinated β-elemene. In order to get the 13-monosubstituted chlorinated β-elemene as the absolutely major product the reaction temperature must be kept below 0 °C, and the reactant NaClO should be fresh and its mole ratio to

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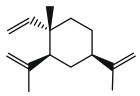
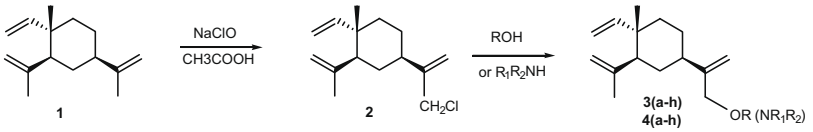


Figure 1. Chemical structure of β-elemene.



3a) R = Me 3b) R = Et 3c) R = Bu 3d) R = t-Bu

3e) R = $(CH_2)_{10}CH_3$, 3f) R = cyclohexyl 3g) R = $PhCH_2$ 3h) R = $CH_2CH_2OCH_3$

4a) R_1R_2 = BuH 4b) R_1R_2 = t-BuH 4c) R_1R_2 = H Cyclohexyl 4d) R_1R_2 = H Cyclopentyl

4e) R_1R_2 = H Cyclooctyl 4f) R_1 R_2 = Imidazole 4g) R_1=R_2=Et 4h) R_1R_2=i-PrH

Scheme 1. Synthetic procedure of β-elemene monosubstituted derivatives.

β-elemene keeps between 1.1 and 1.2, the chlorinated β-elemene mixture was used directly without further purification. In the second step, the 13-monosubstituted β-elemene chloride is more active than the other two chloride products, so when alcohol was added to the chlorinated β-elemene mixture, the reaction mixture was heated and refluxed for about 2 h. After purified with chromatography to give pure β-elemene 13-monosubstituted ether derivatives, Scheme 1 shows the procedure. Under the similar conditions the expected pure 13-monosubstituted amine derivatives cannot be given due to amine is more active than alcohol, the added amine in the second step not only reacts with 13-monosubstituted chloride β-elemene, but also reacts with the other two byproducts. So we had to run the second step at room temperature and luckily got the pure β-elemene 13-monosubstitued amine derivatives after silica gel purification.

For the syntheses of the rhenium coordinated derivatives, we first prepared various chelating systems with the spacer entities according to the literature.[9,10] Their structures were shown in Figure 2.

These intermediates including commercial available compound di-(2-picoyl) amine were coupled to the β-elemene chlorinated compound **2** under basic conditions to form the corresponding intermediates **8, 9, 10** in very good yields.

The complex of $[N(CH_2CH_3)_4]_2[ReBr_3(CO)_3]$ (**7**) was prepared according to a previously published procedure.[9,11] This complex is an important starting material for compounds containing the *fac*-$Re(CO)_3$ moiety since the three bromide ligands are very weak bound. When $[N(CH_2CH_3)_4]_2[ReBr_3(CO)_3]$ complex was dissolved in water, the three bromide ligands were quantitatively exchanged by three H_2O molecules to form the complex of *fac*-$[Re(CO)_3(H_2O)_3]^+$, which is stable in aqueous solution even when exposed to air for several weeks.

These substrates (**8**, **9**, **10)** are easy to coordinate with $[N(CH_2CH_3)_4]_2[ReBr_3(CO)_3]$ to afford the compounds **11, 12** and **13** (Scheme 2). All of them were characterized by IR, ^{1}H NMR, HRMS, HPLC or elemental analysis.

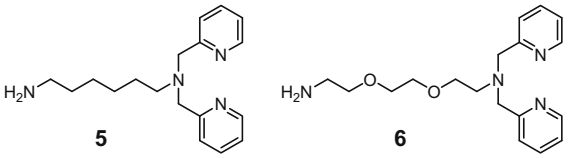


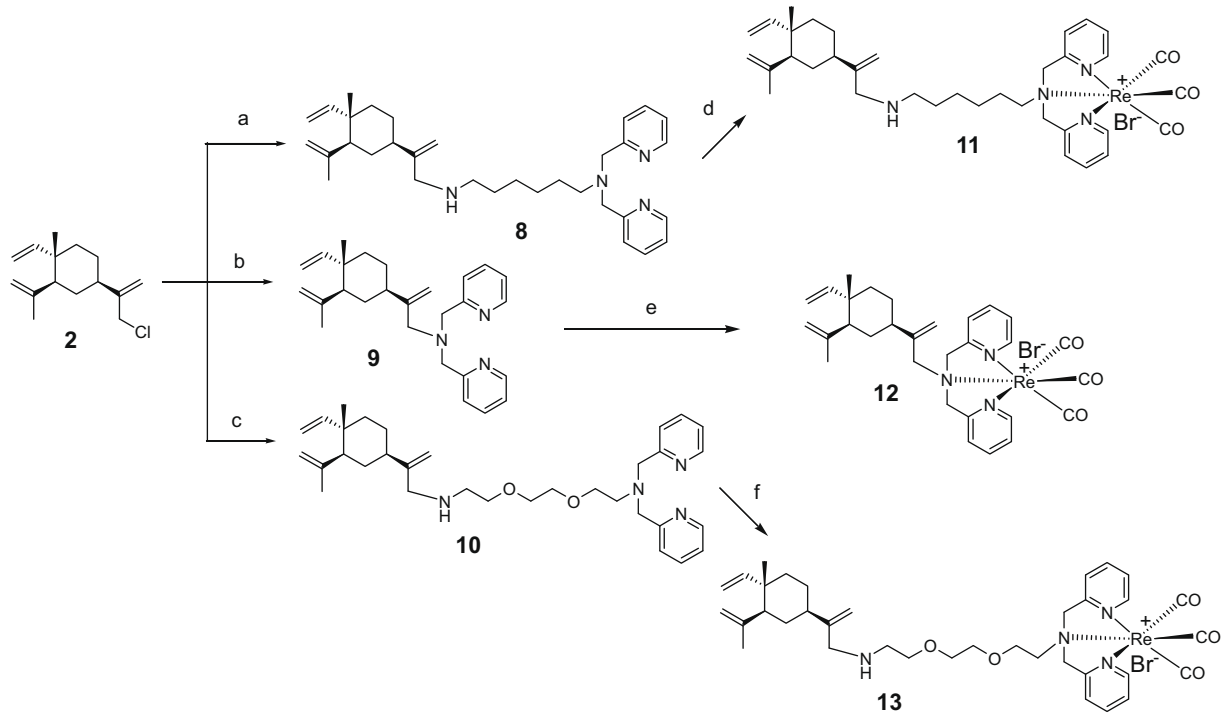
Figure 2. Structure of compound **5** and **6**.

2.2. Anti-proliferative activity

With pure β-elemene derivatives in hands we tested their in vitro antiproliferative activities on HeLa cell lines. Their antiproliferative activities were evaluated by WST-1 method. Results were shown in Table 1, generally speaking the proliferative activity for amine derivatives is much higher than that of the ether derivatives, especially for isopropyl amine, cyclopentyl amine or cyclohexyl amine, their IC_{50} value were decreased dramatically. Compared with the similar work reported by Dong,[7] the IC_{50} for 13,14-disubstituted amine derivatives is similar to our 13-monosubstituted derivative, it seems that β-elemene 14-monosubstituted derivatives contributes extremely weak antiproliferative activities. At the same time, for most of β-elemene ether derivatives, their antiproliferative activity did not increase at all. The methyl, butyl and 2-methoxyl ethyl ether derivatives, their antiproliferative activity is not higher than that of parent β-elemene, the ethyl ether derivative holds a little higher antiproliferative activity. For benzyl, *tert*-butyl and undecyl and cyclohexyl ether derivatives, their IC_{50} value is even higher than that of parent β-elemene (These data was not shown in Table 1). The three rhenium coordinated derivatives shows similar anti-proliferative activities, all of their IC_{50} values decreased significantly compared with parent β-elemene which provides us opportunity for further research other metal coordinated β-elemene derivatives' activity.

FACS data on cell cycle analysis supported the effect of β-elemene and its derivatives on cancer cells proliferation. The results of cell cycle analysis (Fig. 3) indicated the percentage of cells in G_1 phase increased treated by the derivatives, so the percentage of cells in S and G_2-M phase decreased. Therefore the G_1 phase arrest was one of possible mechanisms of anti-proliferative activities for both of β-elemene and its derivatives.

It was found that the cell cycle protein Cyclin D_1 and the cell survival checkpoint protein p-Rb decreased while the protein level of Rb did not decrease (Fig. 4). Cyclin D_1 is an important regulation protein at the G_1/S checkpoint,[12] Rb is an important regulator of the G_1 to S phase transition in the cell cycle and represses gene transcription required for this transition by interacting with E2F transcription factors.[13] Rb is regulated by CDK phosphorylation, and the Rb phosphorylation state continues from the late G_1 phase of the cell cycle until mitosis.[14] The present data showed that onset



Scheme 2. Synthesis of compounds **8–13**. Reagents and conditions: (a) compound **5**, dry CH_3CN, reflux, 8 h; (b) di-(2-picolyl)amine, dry CH_3CN, reflux, 8 h; (c) compound **6**, dry CH_3CN, reflux, 10 h; (d)–(f) compound **7**, CH_3OH, rt, 40 min.

Table 1
Anti-proliferative activity of β-elemene monosubstituted derivatives in HeLa cell lines

Compounds	IC_{50} (μM)
1	236.2 ± 3.2
3a	211.9 ± 2.8
3b	105.9 ± 24.4**
3c	265.9 ± 17.9
3h	237.6 ± 40.3
4a	10.81 ± 0.21**
4b	25.37 ± 0.45**
4c	3.39 ± 1.40**
4d	0.52 ± 0.20**
4e	5.90 ± 2.42**
4f	15.67 ± 1.80**
4g	14.35 ± 1.05**
4h	0.04 ± 0.01**
11	10.9 ± 1.2**
12	11.2 ± 1.5**
13	10.5 ± 2.9**

HeLa cells were treated with β-elemene mono-substituted ether, amine and Re(CO)3-β-elemene derivatives in five different concentrations for 24 h, anti-proliferative activity was evaluated by WST-1 assay, absorbance values were read by a 96-well Opsys Microplate Reader at 450 nm. The IC_{50} were calculated according to the absorbance. Data are presented as mean ± SD, $n = 3$. Differences were considered significant when $p < 0.05$ (*) and $p < 0.01$ (**).

of differentiation initiated Rb phosphorylation. However β-elemene and its derivatives, especially compound **13** reduced the Rb phosphorylation suppress the cell cycle progression at the G_1 checkpoint by interacting with E2F and repressing transcription. These results indicate that β-elemene and its derivatives blocks the cell cycle at the G_1 phase transition and causes HeLa cells to remain at the G_1 phase.

In conclusion, the β-elemene monosubstituted amine, ether and rhenium coordinated derivatives were successfully synthesized for the first time by carefully controlling the reaction conditions and their in vitro anti-proliferative activity were screened by WST-1 method. For monosubstituted amine and rhenium coordinated derivatives their activities were improved significantly, but for monosubstituted ether derivatives their antiproliferative activities are higher than that of parent β-elemene only in some cases. All of them block the cell cycle at the G_1 phase in HeLa cells. These derivatives could reduce Rb phosphorylation and cyclin D_1 protein expression to arrest the cell cycle at G_1 phase which is probably one of the anti-proliferative activities' mechanisms for β-elemene derivatives.

3. Experimental

β-Elemene was obtained from WenTe Research Institute of Oleum Curcumae Wenchowensis in Yue Qing city, Zhejiang province (purity 98%). Other materials were purchased from Fluka Co. and Sinopharm Chemical Reagent Co. Ltds. The NMR data were obtained using a Bruker DRX 500 MHz FT spectrometer. The chemical shifts as δ are reported in ppm relative to TMS. Infrared (IR) spectra were recorded on a Perkin–Elmer FT-IR spectrometer. Mass spectral data were collected using positive mode on a Finnigan LCQ classic mass spectrometer. Elemental analysis was performed using a Perkin–Elmer Series III analyzer.

3.1. Chemistry

3.1.1. A General procedure for the formation of 13-chloro-β-elemene (2)[7]

The formation of **2:** To a solution of β-elemene in glacial acetic acid, sodium hypochlorite was added over 4 h period under stirring at 0 °C, then the mixture was stirred for 2 h at room temperature and extracted with EtOAc. The combined organic extracts were washed with water, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to yield the mixture as yellow oil which was used for the next step directly without further purification.

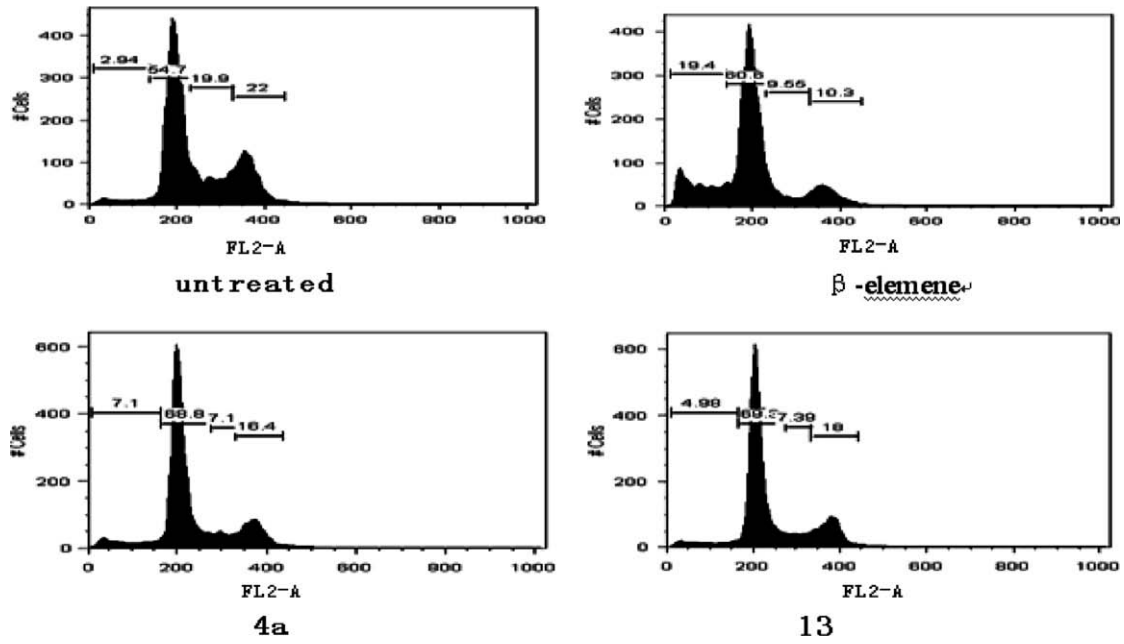


Figure 3. Effects of **4a** and **13** on cell cycle distribution. HeLa cells were treated with compound **4a** and **13** (IC_{20}) for 24 h. DNA content was analyzed by flow cytometry using PI staining, as described in Section 3. The percentage of cells in each phase of the cell cycle was calculated using Cell Quest Pro and is indicated in the top right of each cell cycle profile.

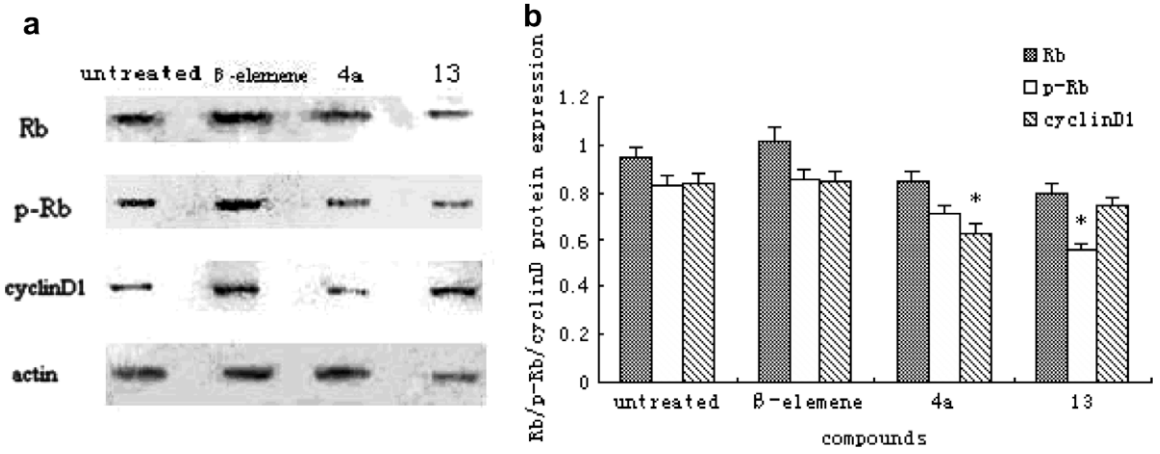


Figure 4. Effects of **4a** and **13** on protein expression of Cyclin D1 and p-Rb. (a) HeLa cells were treated with compound **4a** and **13** (concentration is their IC_{20} values) for 24 h. A total of 50 μg cell extract protein isolated from the drug-treated and untreated HeLa cells was subjected to SDS–PAGE and immunoblotted with antibodies against cyclin D_1, Rb. β-Actin was used as a loading control. (b) The comparative level of Rb, p-Rb and cyclin D1 protein expression were normalized by density ratio of themselves to β-actin on the same lane. Differences were considered significant when $p < 0.05$ (*).

3.1.2. A general procedure for the formation of β-elemene monosubstituted ether derivatives 3

To alcohol solution of β-elemene chloride mixture which contains 13-monosubstituted chloride, 14-monosubstituted chloride and 13,14-disubstituted chloride, 1.5 equiv of base NaOH was added. The mixture was stirred under reflux for 2–6 h, after that the reaction mixture was cooled to rt and washed with water and extracted with EtOAc, dried over anhydrous $MgSO_4$, filtered, concentrated and purified with chromatography to yield the final product 13-monosubstituted ether derivatives **3**.

The formation of compound **3a**: A colorless oil, yield 58%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 0.94 (s, 3H), 1.36–1.71 (m, 6H), 1.64 (s, 3H), 1.94–2.00 (m, 2H), 3.24 (s, 3H), 3.84 (s, 2H), 4.52 (s, 1H), 4.75 (s, 1H), 4.81 (s, 1H), 4.84 (d, J = 3.46, 1H), 4.90 (s, 1H), 4.956 (s, 1H), 5.74 (dd, J = 11.20, 6.47, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 151.2, 150.9, 148.3, 112.8, 110.9, 110.6, 75.7, 58.5, 53.5, 42.0, 40.5, 40.4, 33.9, 27.8, 25.5, 17.3; FTIR (KBr, cm^{-1}): 3081, 2927, 2856, 1641, 1452, 1374, 1107, 1007, 905; EI-HRMS: calcd for $C_{16}H_{27}O$ ($[M+H]^+$ 235.2062, found 235.2063.

The formation of compound **3b**: A colorless oil, yield 64%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.94* (s, 3H), 1.15 (t, J = 7.01, 3H), 1.36–1.44 (m, 3H), 1.49–1.62 (m, 3H), 1.94–2.08 (m, 2H), 3.40 (q, J = 6.97, 2H), 3.89 (s, 2H), 4.52 (s, 1H), 4.75 (s, 1H), 4.82 (s, 1H), 4.85 (d, J = 6.37, 1H), 4.89 (s, 1H), 4.96 (s, 1H), 5.75 (dd, J = 17.36, 11.04, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 147.9, 150.7, 149.5, 111.3, 112.6, 110.5, 73.3, 58.7, 54.0, 41.8, 40.2, 39.3, 33.7, 28.9, 25.3, 16.0, 14.7; FTIR (KBr, cm^{-1}): 3081, 2929, 2856, 1638, 1442, 1375, 1103, 1008, 909; EI-HRMS: calcd for $C_{17}H_{28}O$, requires ($[M+H]^+$ 249.2218; found: M^++H, 249.2219.

The formation of compound **3c**: A colorless oil, yield 42%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ (ppm): 0.85 (t, J = 7.38, 3H), 0.94 (s, 3H), 1.27–1.35 (m, 3H), 1.35–1.42 (m, 3H), 1.44–1.54 (m, 4H),

1.63 (s, 3H), 1.92–2.00 (m, 2H), 3.32 (t, J = 6.58, 2H), 3.87 (s, 2H), 4.51 (s, 1H), 4.74 (s, 1H), 4.81 (s, 1H), 4.84 (d, J = 7.65, 1H), 4.87 (s, 1H), 4.95 (s, 1H), 5.74 (dd, J = 17.37, 10.89, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 149.9, 149.2, 146.6, 111.1, 108.7, 107.3, 72.1, 69.0, 51.8, 40.7, 40.4, 38.7, 32.2, 30.9, 26.1, 25.7, 15.6, 15.0, 12.9; FTIR (KBr, cm^{-1}): 3081, 2930, 2862, 1640, 1457, 1375, 1102, 1006, 904; EI-HRMS: calcd for $C_{19}H_{33}O$ requires ($[M+H]^+$, 277.2531; found: M^++H, 277.2539.

The formation of compound **3d**: A colorless oil, yield 48%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.94* (s, 3H), 1.19 (s, 9H), 1.34–1.41 (m, 3H), 1.46–1.51 (m, 3H), 1.64 (s, 3H), 1.92–2.00 (m, 2H), 3.88 (d, J = 3.74, 2H), 4.52 (s, 1H), 4.75 (s, 1H), 4.82 (s, 1H), 4.85 (d, J = 6.64, 1H), 4.90 (s, 1H), 4.97 (s, 1H), 5.75 (dd, J = 17.45, 11.17, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 151.4, 150.9, 148.3, 112.8, 110.8, 110.6, 77.5, 73.0, 53.5, 42.1, 40.7, 40.5, 32.6, 30.4, 30.0, 25.5, 17.3; FTIR (KBr, cm^{-1}): 3080, 2924, 2853, 1640, 1463, 1375, 1080, 905; EI-HRMS: calcd for $C_{19}H_{33}O$ ($[M+H]^+$, 277.2531; found: M^++H, 277.2533.

The formation of compound **3e**: A colorless oil, yield 47%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.88* (t, J = 6.57, 3H), 1.01 (s, 1H), 1.20–1.1.38 (m, 20H), 1.41–1.65 (m, 6H), 1.71 (s, 3H), 1.99–2.09 (m, 2H), 3.39 (t, J = 6.60, 2H), 3.95 (s 2H), 4.59 (s, 1H), 4.88 (s, 1H), 4.92 (d, J = 7.76, 2H), 4.94 (s, 1H), 5.82 (dd, J = 17.37, 10.95, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 146.5, 149.8, 149.2, 111.1, 108.9, 107.3, 72.1, 69.3, 51.8, 40.4, 39.0, 38.8, 32.2, 30.9, 28.70, 28.64, 28.63, 28.58, 28.55, 28.4, 26.1, 25.1, 23.8, 21.7, 15.6, 13.1; FTIR (KBr, cm^{-1}): 3081, 2926, 2854, 1640, 1461, 1375, 1106, 1005, 905; EI-HRMS: calcd for $C_{26}H_{47}O$ ($[M+H]^+$, 375.3627; found: M^++H, 375.3618.

The formation of compound **3f**: A colorless oil, yield 50%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.94* (s, 1H), 1.10–1.26 (m, 6H), 1.34–1.48 (m, 4H), 1.50–1.54 (m, 2H), 1.58–1.72 (m, 2H), 1.80–1.86 (m, 2H), 1.93–2.02 (m, 2H), 3.16–3.20 (m, 1H), 3.92 (s, 2H), 4.52 (s, 1H), 4.75 (s, 1H), 4.82 (s, 1H), 4.83–4.86 (m, 2H), 4.98 (s, 1H), 5.75 (dd, J = 17.35, 10.98, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 152.2, 151.0, 148.3, 112.8, 110.5, 110.0, 77.4, 70.7, 53.5, 42.2, 40.5, 40.4, 33.9, 32.9, 27.8, 26.5, 25.4, 24.8, 17.3; FTIR (KBr, cm^{-1}): 3080, 2930, 2856, 1640, 1450, 1373, 1088, 1006, 904; EI-HRMS: calcd for $C_{21}H_{34}O$, requires ($[M+H]^+$ 303.2688; found: M^++H, 303.2684.

The formation of compound **3g**: A colorless oil, yield 53%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.93* (s, 3H), 1.36–1.43 (m, 3H), 1.49–1.56 (m, 3H), 1.63 (s, 3H), 1.94 (dd, J = 11.82, 4.07, 1H), 1.99–2.06 (m, 1H), 3.95 (s, 2H), 4.42 (s 2H), 4.51 (s, 1H), 4.75 (s, 1H), 4.81 (s 1H), 4.84 (d, J = 6.95, 1H), 4.92 (s, 1H), 5.01 (s, 1H), 5.75 (dd, J = 17.17, 11.16), 7.25–7.29 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 151.2, 150.8, 148.2, 139.1, 129.0, 128.3, 128.1, 112.8, 111.1, 110.5, 73.1, 72.6, 53.4, 42.1, 40.6, 40.4, 33.8, 30.4, 27.7, 17.3; FTIR (KBr, cm^{-1}): 3081, 2926, 2854, 1640, 1453, 1374, 1093, 1007, 905, 734, 697; EI-HRMS: calcd for $C_{22}H_{31}O$ ($[M+H]^+$, 311.2375; found: M^++H, 311.2372.

The formation of compound **3h**: A colorless oil, yield 46%; ^{1}H NMR ($CDCl_3$, TMS, 400 MHz), δ *(ppm): 1.01 (s, 3H), 1.41–1.53* (m, 3H), 1.55–1.74 (m, 3H), 1.71 (s, 3H), 1.99–2.09 (m, 2H), 3.39 (s, 3H), 3.55 (s, 4H), 4.02 (s, 2H), 4.59 (s, 1H), 4.82 (s, 1H), 4.88 (s, 1H), 4.92 (d, J = 6.35, 1H), 4.97 (s, 1H), 5.03 (s, 1H), 5.82 (dd, J = 17.34, 10.98, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 149.4, 148.7, 146.6, 111.1, 109.3, 107.3, 72.6, 71.0, 68.0, 58.0, 51.7, 40.2, 38.9, 38.7, 32.1, 26.1, 23.8, 15.6; FTIR (KBr, cm^{-1}): 3080, 2927, 2868, 1638, 1456, 1374, 1115, 1008, 905, 734, 697; EI-HRMS: calcd for $C_{18}H_{31}O_2$ ($[M+H]^+$, 279.2324; found: M^++H, 279.2281.

3.1.3. A general procedure for the formation of β-elemene monosubstituted amine derivatives 4

To CH_3CN solution of β-elemene chloride mixture which contains 13-monosubstituted chloride, 14-monosubstituted chloride and 13,14-disubstituted chloride, 1.5 equiv of NaOH was added followed by 1.2 equiv of $BuNH_2$. The mixture was stirred at room temperature for 3 h, then washed with water and extracted with EtOAc, dried over anhydrous $MgSO_4$, filtered, concentrated and purified with chromatography to yield the final product 13-monosubstituted amine derivatives.

The formation of compound **4a**: A yellow oil, yield 48%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.92* (t, J = 7.32 Hz, 3H), 1.01 (s, 3H), 1.36–1.74 (m, 10H), 1.71 (s, 3H), 2.00–2.10 (m, 2H), 2.68 (t, J = 7.49 Hz, 2H), 3.33 (s, 2H), 4.06 (s, 1H, NH), 4.59 (s, 1H), 4.82 (s, 1H), 4.89 (s, 1H), 4.91 (d, J = 7.78 Hz, 1H), 4.99 (s, 1H), 5.04 (s, 1H), 5.82 (dd, J = 17.37, 10.94 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 150.6, 148.4, 148.0, 112.8, 110.7, 109.1, 53.0, 52.3, 48.3, 42.7, 40.4, 40.3, 33.7, 30.2, 27.8, 25.6, 20.9, 17.2, 14.3; FTIR (KBr, cm^{-1}): 3081, 2927, 2856, 1641, 1452, 1374, 1107, 1007, 905; MS (EI+) calcd for $C_{19}H_{33}N$ [M^+] 275, found 275.

The formation of compound **4b**: A yellow oil, yield 43%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.99* (s, 3H), 1.41–1.64 (m, 6H), 1.49 (s, 9H), 1.71 (s, 3H), 2.12 (dd, J = 12.76, 3.08 Hz, 1H), 2.28–2.40 (m, 1H), 3.52 (q, J = 13.67 Hz, 2H), 4.57 (s, 1H), 4.82 (s, 1H), 4.89 (s, 1H), 4.92 (d, J = 7.05 Hz, 1H), 5.83 (dd, J = 17.23, 11.17 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 150.5, 148.0, 145.3, 114.8, 112.8, 110.7, 58.5, 52.9, 45.7, 42.7, 40.3, 40.2, 27.8, 27.5, 26.5, 21.8, 17.1; FTIR (KBr, cm^{-1}): 3434, 3080, 2929, 2757, 1637, 1442, 1377, 1008, 907; MS (EI+) calcd for $C_{19}H_{33}N$ [M^+] 275, found 275.

The formation of compound **4c**: A yellow oil, yield 36%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.99* (s, 3H), 1.17–1.30 (m, 4H), 1.35–1.59 (m, 10H), 1.71 (s, 3H), 1.78–1.90 (m, 2H), 2.05–2.26 (m, 2H), 2.91–3.02 (m, 1H), 3.50–3.66 (m, 2H), 4.57 (s, 1H), 4.82 (s, 1H), 4.88 (s, 1H), 4.90–4.92 (m, 1H), 5.23 (s, 1H), 5.35 (s, 1H), 5.82 (dd, J = 17.76, 11.1 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 149.9, 147.4, 144.5, 112.2, 110.1, 108.5, 56.5, 53.8, 52.2, 41.7, 39.7, 39.6, 32.9, 29.0, 27.1, 25.1, 25.0, 24.7, 16.6; FTIR (KBr, cm^{-1}): 3395, 3079, 2934, 2858, 1637, 1454, 1375, 1007, 907; MS (EI+) calcd for $C_{21}H_{35}N$ [M^+] 301, found 301.

The formation of compound **4d**: A yellow oil, yield 42%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 1.01* (s, 3H), 1.42–1.60 (m, 10H), 1.67–1.75 (m, 4H), 1.71 (s, 3H), 1.83–1.92 (m, 2H), 1.98–2.15 (m, 2H), 3.15–3.20 (m, 1H), 3.30 (s, 2H), 4.59 (s, 1H), 4.82 (s, 1H), 4.89 (s, 1H), 4.91 (d, J = 7.04 Hz, 1H), 4.95 (s, 1H), 5.00 (s, 1H), 5.84 (dd, J = 17.43, 11.05 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 153.6, 150.9, 148.3, 112.8, 110.5, 108.5, 60.0, 53.5, 52.9, 43.4, 40.7, 40.4, 33.8, 28.0, 25.4, 24.8, 21.7, 17.3; FTIR (KBr, cm^{-1}): 3419, 3080, 2930, 2862, 1638, 1440, 1375, 1006, 907; MS (EI+) calcd for $C_{20}H_{33}N$ [M^+] 287, found 287.

The formation of compound **4e**: A yellow oil, yield 58%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 1.01* (s, 3H), 1.40–1.65 (m, 16H), 1.67–1.81 (m, 4H), 1.71 (s, 3H), 1.98–2.06 (m, 2H), 2.69–2.77 (m, 1H), 3.27 (s, 2H), 4.59 (s, 1H), 4.82 (s, 1H), 4.88 (s, 1H), 4.89–4.94 (m, 2H), 4.97 (s, 1H), 5.82 (dd, J = 17.41, 10.95 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 149.6, 148.9, 147.4, 111.9, 111.7, 110.0, 57.4, 53.7, 52.3, 42.1, 39.7, 39.6, 33.2, 31.1, 26.7, 26.5, 25.9, 24.9, 24.0, 16.5; FTIR (KBr, cm^{-1}): 3447, 3080, 2923, 2855, 1638, 1445, 1375, 1006, 906; MS (EI+) calcd for $C_{23}H_{39}N$ [M^+] 329, found 329.

The formation of compound **4f**: A yellow oil, yield 48%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.99* (s, 3H), 1.41–1.48 (m, 3H), 1.53–1.64 (m, 3H), 1.70 (s, 3H), 1.82–1.88 (m, 1H), 1.93–1.98 (m, 1H), 4.53 (s, 2H), 4.57 (s, 1H), 4.59 (s, 1H), 4.83 (s, 1H), 4.88 (d, J = 2.42 Hz, 1H), 4.91 (d, J = 2.63 Hz, 1H), 5.04 (s, 1H), 5.77 (dd, J = 17.71, 10.52 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 150.4, 150.1, 147.9, 138.2, 123.0, 120.0, 113.0, 112.4, 110.9, 54.5, 53.2, 42.2, 40.4, 40.3, 33.9, 27.8, 25.5, 16.3; FTIR (KBr, cm^{-1}): 3398, 3080, 2929, 2859, 1637, 1504, 1439, 1375, 1008, 905; MS (EI+) calcd for $C_{18}H_{26}N_2$ [M^+] 270, found 270.

The formation of compound **4g**: A yellow oil, yield 40%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ *(ppm): 0.99* (t, J = 7.05 Hz, 6H), 1.01 (s,

3H), 1.41–1.65 (m, 6H), 1.71 (s, 3H), 2.02–2.10 (m, 2H), 2.45 (t, J = 7.05 Hz, 4H), 2.96 (s, 2H), 4.59 (s, 1H), 4.81 (s, 1H), 4.88 (s, 2H), 4.91 (d, J = 8.94 Hz, 1H), 4.95 (s, 1H), 5.83 (dd, J = 17.44, 10.86 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 153.4, 151.1, 148.6, 112.6, 110.4, 108.9, 59.1, 53.4, 47.4, 42.4, 40.8, 40.5, 34.0, 28.0, 25.5, 17.3, 12.3; FTIR (KBr, cm^{-1}): 3432, 3081, 2931, 2869, 1639, 1454, 1375, 1004, 906; MS (EI+) calcd for $C_{19}H_{33}N$ [M^+] 275, found 275.

The formation of compound **4h**: A yellow oil, yield 51%; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), *δ (ppm): 1.00* (s, 3H), 1.17 (d, J = 6.12 Hz, 6H), 1.42–1.76 (m, 6H), 1.71 (s, 3H), 1.99–2.17 (m, 2H), 2.96–3.05 (m, 1H), 3.36 (s, 2H), 4.36 (s, 1H, NH), 4.58 (s, 1H), 4.71 (s, 1H), 4.88 (s, 1H), 4.90–4.95 (m, 1H), 4.97 (s, 1H), 5.03 (s, 1H), 5.83 (dd, J = 18.67, 11.13 Hz, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 150.0, 147.5, 145.1, 114.9, 112.3, 110.2, 52.2, 50.2, 48.0, 41.9, 39.8, 39.7, 33.1, 27.1, 25.1, 19.5, 16.7; FTIR (KBr, cm^{-1}): 3423, 3081, 2929, 2862, 1638, 1439, 1378, 1006, 906; MS (EI+) calcd for $C_{19}H_{31}N$ [M^+] 261, found 261.

3.1.4. General procedure for the preparation of compounds 8–10

A solution of Cl-β-elemene**,** (2 mmol), **5** or Di-(2-picolyl) amine or **6** (4 mmol), and sodium hydroxide (8 mmol) in 10 mL dry acetonitrile was refluxed for 8–10 h. Then water (10 mL) was added and the mixture was extracted with ethyl ether (4 × 30 mL). The combined organic extracts were dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated in vacuo. The residue was purified on a silica gel column with dichloromethane–methanol as eluent to give a target product.

The formation of compound **8**: A brown oil, 80% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 0.97 (s, 3H), 1.19–1.32 (m, 4H), 1.35–1.60 (m, 8H), 1.62–1.75 (m, 2H), 1.69 (s, 3H), 1.96–2.12 (m, 2H), 2.55 (t, J = 6.88 Hz, 2H), 2.89 (t, J = 7.78 Hz, 2H), 3.61 (s, 2H), 3.84 (s, 4H), 4.55 (s, 1H), 4.80 (s, 1H), 4.87–4.94 (m, 2H), 5.15 (s, 1H), 5.17 (s, 1H), 5.79 (dd, ^1J = 17.71 Hz, ^2J = 10.54 Hz, 1H), 7.17 (t, J = 5.6 Hz, 2H), 7.50 (d, J = 7.79 Hz, 2H), 7.67 (dt, ^1J = 7.67 Hz, ^2J = 1.64 Hz, 2H), 8.53 (d, J = 4.19 Hz, 2H); FTIR (KBr, cm^{-1}): 2928, 2362, 1388; ESI-HRMS calcd for $C_{33}H_{49}N_4$ ([M+H])$^+$ 501.3957, found 501.3922.

The formation of compound **9**: A brown oil, 87% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 0.90 (s, 3H), 1.47–1.27 (m, 6H), 1.61 (s, 3H), 1.92–1.89 (m, 1H), 2.07–2.04 (m, 1H), 3.06 (q, 2 H, J = 14.0 Hz), 3.73 (q, J = 14.1 Hz, 4H), 4.47 (s, 1H), 4.73 (s, 1H), 4.84 (dd, J = 5.82 Hz, 1.08 Hz, 1H), 4.85 (d, J = 1.3 Hz, 1H), 4.89 (s, 1H), 5.06 (s, 1H), 5.77 (dd, J = 10.48 Hz, 7.38 Hz, 1H), 7.09–7.07 (m,H), 7.50–7.47 (m,H), 7.60 (dt, J = 7.65 Hz, 1.57 Hz, 2H), 8.45 (d, J = 4.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, TMS, 125 MHz), δ: 17.39, 25.31, 27.68, 33.73, 40.57, 40.74, 42.56, 53.74, 59.71, 60.80, 110.53, 111.63, 112.71, 122.62, 123.41, 137.10, 148.14, 149.61, 150.94, 152.06, 160.46; FTIR (KBr, cm^{-1}): 3080, 1640; ESI-HRMS calcd for $C_{27}H_{35}N_3$ ([M+H])$^+$ 401.2831, found 401.2842.

The formation of compound 10: A brown oil, 81% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 0.97 (s, 3H), 1.35–1.63 (m, 6H), 1.67 (s, 3H), 1.97–2.16 (m, 2H), 2.89 (s, 2H), 3.22 (t, J = 4.32 Hz, 2H), 3.46–3.51 (m, 2H), 3.57–3.65 (m, 4H), 3.69 (s, 2H), 3.81–3.95 (m, 6H), 4.54 (s, 1H), 4.80 (s, 1H), 4.86–4.94 (m, 2H), 5.18 (s, 1H), 5.32 (s, 1H), 5.78 (dd, ^1J = 17.85 Hz, ^2J = 6.85 Hz, 1H), 7.17 (t, J = 6.65 Hz, 2H), 7.38 (d, J = 7.45 Hz, 2H), 7.66 (t, J = 7.64 Hz, 2H), 8.55 (d, J = 3.97 Hz, 2H); FTIR (KBr, cm^{-1}): 3371, 2928, 1639, 1591, 765; ESI-HRMS calcd for $C_{33}H_{49}N_4O_2$ ([M+H])$^+$ 533.3856, found 533.3845.

The synthesis of compound **5** and **6** according to literature.[9,10]

Compound **5:** A colorless oil, 92% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 1.20–1.29 (m, 4H), 1.35–1.41 (m, 2H), 1.50–1.55 (m, 2H), 1.75 (s, 2H), 2.52 (t, J = 7.29, 2H), 2.62 (t, J = 7.06 Hz, 2H), 3.79 (s, 4H), 7.12 (t, J = 5.20 Hz, 2H), 7.52 (d, J = 7.80 Hz, 2H), 7.63 (dt, ^1J = 7.71 Hz, ^2J = 1.66 Hz, 2H), 8.50 (d, J = 4.13 Hz, 2H); FTIR (KBr, cm^{-1}): 2924, 2028, 1917; EI-HRMS: calcd for $C_{18}H_{26}N_4$ ([M+H])$^+$ 298.2157, found 298.2157.

Compound **6**: A colorless oil, 87% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 2.64 (s, 2H), 2.76–2.85 (m, 4H), 3.48–3.68 (m, 8H), 3.87 (s, 4H), 7.12 (t, J = 6.40 Hz, 2H), 7.51 (d, J = 7.60 Hz, 2H), 7.62 (dt, ^1J = 7.60 Hz, ^2J = 1.60 Hz, 2H), 8.49 (d, J = 4.80 Hz, 2H); FTIR (KBr, cm^{-1}): 3458, 2987, 1999, 1858; ESI-HRMS: calcd for $C_{18}H_{27}N_4O_2$ ([M+H])$^+$ 331.2134, found 331.2123.

3.1.5. General procedure for the preparation of compounds 11–13

Complexes **11–13** were prepared according to the following general procedure: 2 mmol $[N(CH_2CH_3)_4]_2[ReBr_3(CO)_3]$, and 2 mmol corresponding derivative (**8–10**) was dissolved in CH_3OH and stirred for 30 min. The mixture was evaporated, and dried in a vacuum and the desired product was recrystallized with n-hexane/dichloromethane = 1:2.

The formation of compound **11**. A white solid, 78% yield; mp 161.7–162.1 °C; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 0.99 (s, 3H), 1.23–1.33 (m, 2H), 1.38–1.46 (m, 2H), 1.47–1.59 (m, 3H), 1.62–1.83 (m, 4H), 1.70 (s, 3H), 2.10–2.21 (m, 4H), 2.27–2.33 (m, 1H), 3.05 (t, J = 7.16 Hz, 2H), 3.70 (q, J = 14.00 Hz, 2H), 3.81 (t, J = 8.08 Hz, 2H), 4.57 (s, 1H), 4.73 (d, J = 16.75 Hz, 2H), 4.81 (s, 1H), 4.87–4.89 (m, 1H), 4.91 (s, 1H), 5.26 (s, 1H), 5.45 (s, 1H), 5.59 (d, J = 17.02 Hz, 2H), 5.82 (dd, J = 17.49 Hz, 10.85, 1H), 7.23 (t, J = 6.71 Hz, 2H), 7.84 (dt, J = 7.74 Hz, 0.38, 2H), 7.93 (d, J = 7.81 Hz, 2H), 8.67 (d, J = 5.4 Hz, 2H); ^{13} C NMR (CD_3OD, 125 MHz), δ: 197.6, 196.7, 162.5, 153.4, 151.5, 148.9, 147.4, 141.9, 129.7, 127.1, 114.6, 113.2, 110.9, 73.2, 72.0, 71.6, 69.1, 59.4, 54.1, 52.3, 48.3, 43.9, 41.2, 41.0, 34.6, 31.0, 27.5, 27.3, 26.2, 25.6, 17.4; FTIR (KBr, cm^{-1}): ν: 3472, 2926, 2027, 1912 cm^{-1}; Anal. Calcd for $C_{36}H_{48}BrN_4O_3Re{\cdot}CH_2Cl_2$: C, 47.49; H, 5.39; N, 5.99. Found: C, 47.29; H, 5.64; N, 6.26.

The formation of compound **12**. A white solid, 82% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 1.03 (s, 3 H, CH_3), 1.51–1.58 (m, 3H), 1.68 (s, 3 H, CH_3), 1.72–1.82 (m, 2H), 1.97–2.02 (m, 1H), 2.17–2.19 (m, 1H), 2.30–2.39 (m, 1H), 4.36–4.49 (m, 4H), 4.61 (s, 1H), 4.85 (s, 1H), 4.91–4.99 (m, 2H), 5.53 (s, 1H), 5.62 (s, 1H), 5.77–5.89 (q, 1H), 6.05 (q, J = 18.53 Hz, 2H), 7.18 (t, J = 6.39 Hz, 2H), 7.80 (t, J = 7.63 Hz, 2H), 8.00 (d, J = 7.64 Hz, 2H), 8.63 (d, J = 5.34 Hz, 2H); FTIR (KBr, cm^{-1}): 3080, 2027, 1910 cm^{-1}; ^{13}C NMR ($CDCl_3$, TMS, 125 MHz), δ: 17.23, 25.64, 28.00, 34.65, 40.44, 40.82, 44.52, 52.92, 67.78, 67.92, 74.48, 110.86, 113.00, 121.66, 125.85, 126.36, 140.96, 147.12, 148.02, 150.49, 151.07, 161.84, 161.95, 196.22, 196.86; Anal. Calcd for $C_{30}H_{36}BrN_4O_3Re{\cdot}CH_2Cl_2$: C, 46.99; H, 4.73; N, 7.31. Found: C, 46.63; H, 4.69; N, 7.08.

The formation of compound **13**. A white solid, 75% yield; ^{1}H NMR ($CDCl_3$, TMS, 500 MHz), δ: 1.00 (s, 3H), 1.38–1.47 (m, 2H), 1.48–1.61 (m, 2H), 1.74 (s, 3H), 1.65–1.93 (m, 10H), 2.08–2.31 (m, 6H), 3.01–3.09 (m, 2H), 3.62–3.74 (m, 2H), 3.76–3.85 (m, 2H), 4.58 (s, 1H), 4.73 (d, J = 16.80 Hz, 2H), 4.81 (s, 1H), 4.87 (s, 1H), 4.91 (d, J = 8.8 Hz, 1H), 5.27 (s, 1H), 5.48 (s, 1H), 5.64 (d, J = 16.80 Hz, 2H), 5.82 (dd, J = 17.49 Hz, 10.85, 1H), 7.23 (t, J = 6.80 Hz, 2H), 7.84 (t, J = 7.60 Hz, 2H), 7.95 (d, J = 7.60 Hz, 2H), 8.68 (d, J = 6.80 Hz, 2H); ^{13}C NMR (CD_3OD, 125 MHz), δ: 197.6, 196.8, 162.5, 155.6, 153.5, 148.9, 143.2, 141.9, 127.2, 125.0, 124.9, 113.2, 113.0, 99.4, 72.1, 72.0, 69.1, 62.0, 44.0, 41.2, 41.0, 36.1, 31.1, 28.8, 27.7, 27.3, 26.6, 26.4, 25.6, 17.5; FTIR (KBr, cm^{-1}): ν: 1914, 2028, 2928, 3437 cm^{-1}. Anal. Calcd for $[C_{36}H_{48}N_4O_5Re]Br{\cdot}1/3CH_2Cl_2$: C, 47.89; H, 5.38; N, 6.15. Found: C, 47.80; H, 5.45; N, 6.11.

3.2. Cell culture and cytotoxicity essay

HeLa cell line (human uterine cervix carcinoma cells) were obtained from Shanghai Cell Repository of Chinese Academy of

Sciences. cells were maintained in RPMI 1640(Gibico) with 10% inactivated fetal bovine serum(Gibico), penicillin (100 IU/mL) and streptomycin (100 μg/mL), and L-glutamine. The cell lines were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

The HeLa cells were harvested using 0.25% trypsin-EDTA and seeded 5×10^3 cells per well of a 96-well plate and incubated for 12 h. Then the β-elemene monosubstituted amines, ether and PEGylation derivatives were added to wells of the plate at five different concentrations and continued to culture in CO_2 incubator for 24 h, respectively. 10 μL WST-1[2- (4-lodophenyl)-3(4-nitrophenyl)-5-(2,4-disulfonphenyl)-2*H*-tetrazolium·Na] solution was added to wells, absorbance values were taken using a 96-well Opsys Microplate Reader at 450 nm.[15] IC_{50} were calculated according to absorbance.

IC_{50} were calculated with SPSS 11.5 Statistical software. Data are presented as mean ± SD.

3.3. Cell cycle analysis

HeLa cells were incubated with β-elemene monosubstituted amines, ether, Rhenium coordinated derivatives and β-elemene (IC_{20}) for 24 h at 37 °C. Cells suspensions from either control cultures or drug-treated cultures were prepared by trypsinization. Approximately 1×10^6 Cells were washed twice with cold PBS, fixed in chilled methanol (75%) at −20 °C overnight. Then ethanol was removed by centrifuging the cells and washing them with PBS. The cells were then incubated in 0.1% Triton X-100-containing 1 mg/mL RNase on ice for 30 min, then stained with 50 μg/mL propidine iodide (PI). Cell cycle analysis was performed by flow cytometry (FACScan).

3.4. Western blot analysis

This method was used according to the method of Janecka et al. with slightly modification.[16,17] HeLa cells treated with β-elemene, the monosubstituted amines, derivatives and the Rhenium coordinated derivatives which were harvested by trypsinization followed by 24 h incubation. After wash with ice-cold PBS, the cells were lysed on ice in RIPA buffer, containing 10 ml/ml 200 mM phenylmethylsulfonyl fluoride (PMSF). Cellular extracts were clarified by centrifugation at 12,000 rpm at 4 °C for 15 min, and protein concentrations were determined using the Bradford assay. Cell lysate (50 μg) was separated by 12% SDS–PAGE and transferred to PVDF membranes. Blots were incubated with blocking buffer (0.1 M Tris–HCl, PH 7.4, 0.9% NaCl, 0.1% Tween 20 and 5% milk) for 2 h at room temperature. Then, the rabbit polyclonal anti-Cycin D1and anti-Rb antibody (Santa Cruz Biotechnology, Inc.) at 1:100 dilution in 5% milk solution in TTBS was added and incubated with PVDF for 1 h at 37 °C with constant shaking. After three 15 min wash washes in TTBS, the PVDF membranes were incubated for 1 h at 37 °C with secondary antibody (goat anti-rabbit biotynylated IgG) at 1:400 dilution. After being washed three times in TTBS, PVDF membranes were incubated in ABC (Boster Biological Technology. Ltd) reagent for 30 min. When the color developed, the membranes were washed extensively with distilled water.

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