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Original article

Cytotoxicity against cholangiocarcinoma cell lines of zerumbone derivatives

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ABSTRACT

Cholangiocarcinoma (CCA) is an aggressive malignancy with a very high morbidity and mortality for which an effective treatment is lacking. In this study, seventeen zerumbone derivatives were synthesized and evaluated for in vitro cytotoxicity against cholangiocarcinoma cell lines. **5** showed the most potent antiproliferative activity against KKU-100 cell line with an IC_{50} value of 16.44 μM. To investigate the potential molecular target of the most active compound, the docking was performed using different enzymes and receptor proteins including CDK-2, CDK-5, EGFR, and GSK-3. The docking results revealed that **5** exhibited better binding interaction to EGFR than CDK-2, CDK-5 and GSK-3. All results indicate that **5** should be a promising candidate for treatment of cancer.

1. Introduction

Cholangiocarcinoma (CCA) is a primary cancer of the bile duct epithelial cells. It is one of the most serious and highest incidence diseases in the northeast of Thailand where the prevalence of *Opisthorchis viverrini* (OV) infection is high [1]. An increasing trend of CCA incidence has been reported in several countries in the world [2]. This tumor continues to be associated with poor prognosis [1]. At present, surgical resection is the best treatment available and is a potentially curative therapy for CCA. However, more than half CCA patients present with advanced, unresectable malignancy. In addition, the response of CCA to chemotherapy and radiotherapy is relatively poor [3]. At present, there is no effective chemotherapy regimen for treating patients with advanced cholangiocarcinoma. Therefore, novel and effective therapeutic agents and more effective medical treatment options are urgently needed.

Much attention has been focused on the targeted medical therapy. Recently, the status and future perspectives of anti-angiogenic and growth factor receptor-based pharmacological approaches for the treatment of biliary tract cancer have been reported. Among the cellular molecules, the epidermal growth factor receptor (EGFR) and protein kinases are found to play an important role in cell cycle progression, cell proliferation and induction of apoptosis [4].

Medicinal plants which also show promising effects in treatment of cancers are now attracting great attention in the world. The rhizomes of *Zingiber zerumbet* Smith are employed as traditional medicine in relieving stomach ache, as a diuretic, and when macerated in alcohol are regarded as a tonic and depurative. Moreover, it is also used as the spice ginger and a novel food factor for mitigating experimental ulcerative colitis [5].

Zerumbone, a crystalline sesquiterpene, is the major component in the rhizomes of this plant and is readily available from a widespread natural source. It contains three double bonds; an isolated one at C6 and two double bonds at C2 and C10 which are part of a cross conjugated dienone system. It was found that the C2 double bond appears least hindered due to being furthest from the *gem* dimethyl substituents at C9. It has been reported that the X-ray structure shows that the dienone system lies in a slightly distorted plane which is perpendicular to that of the isolated double bond [6].

Zerumbone has been studied for various biological activities because it acts to suppress tumor promoters [7] and to inhibit the growth of a human leukemia cell line [8]. It is also antiproliferative and an anti-platelet activating factor [9,10], and has anti HIV activities [11]. Thus, it is expected that the zerumbone derivatives

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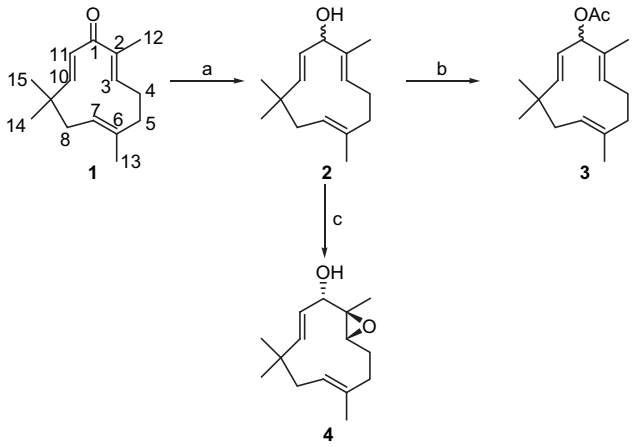
might show useful biological activities, especially cytotoxicity against CCA cell lines.

Kitayama and coworkers [12–15] have reported the isolation and structural transformation of zerumbone as well as the regio- and stereochemistry of its derivatives. Due to this chemical information and as part of our research program on anti-tumor drugs from natural products, we planned to synthesize known and new derivatives of zerumbone and evaluate them for in vitro cytotoxicity against CCA cell lines including KKU-100 (poorly-differentiated adenocarcinoma), KKU-M139 (squamous cell carcinoma), KKU-M156 (moderately-differentiated adenocarcinoma), KKU-M213 (adenosquamous carcinoma) and KKU-M214 (moderately-differentiated adenocarcinoma) cell lines [16]. It is expected that this work will find potent compounds for the development of anti-CCA agents. In addition, to understanding the mode of reaction, the interaction between the most active compound and the enzymes or receptor proteins involved with cell cycle and cell growth was studied using molecular modeling techniques.

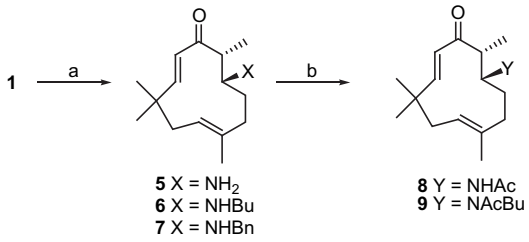
2. Results and discussion

2.1. Chemistry

Seventeen zerumbone derivatives were successfully prepared using organic reactions. Reduction of zerumbone (**1**) using $LiAlH_4$ at 0 °C gave crystalline (±) zerumbol (**2**) [12]. Acetylation of this compound using Ac_2O/pyridine afforded **3** while epoxidation of **2** with *m*CPBA yielded racemic **4** (Scheme 1). The relative stereochemistry of hydroxyl and epoxide groups was *trans* configuration. Zerumbone was stirred with excess ammonia, butylamine and benzylamine at room temperature to provide monoamines **5**, **6** and **7**, respectively. This conjugated amination yielded a single diastereomer as relatively *trans* configuration adducts of each monoamine (Scheme 2). As in a previous report [13], no diamine and no another diastereomer of products was observed. Michael addition of amines occurred selectively at the less hindered conjugated double bond (C2–C3) of zerumbone. Acetylation of amine groups at C3 position of **5** and **6** using Ac_2O/pyridine at 0 °C provided the corresponding amides **8** and **9**, respectively. The reaction of butylamine **6** with $NaBH_4$ afforded hydroxyamine **10** as a single diastereoisomer in 81% yield. The reduction of amide **8** with $NaBH_4$ at 0 °C afforded a single diastereoisomer hydroxyamide **11** in the yield of 54%. The NOESY experiment showed the correlation of protons at C1 and C2 which indicated the relatively *cis* orientation of these positions (Scheme 3).



Scheme 1. Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C, 1 h, 88%; (b) Ac_2O, pyridine, reflux, 30 min, 74%; (c) *m*CPBA, EtOAc, rt, 24 h, 15%. **Note**: The stereochemistry shown in all schemes are relative configuration.



Scheme 2. Reagents and conditions: (a) NH_3 or $BuNH_2$ or $BnNH_2$, MeCN, rt, 5 days, 56% (**5**), 93% (**6**), 68% (**7**); (b) Ac_2O, pyridine, 0 °C, 8 h, 92% (**8**), 85% (**9**).

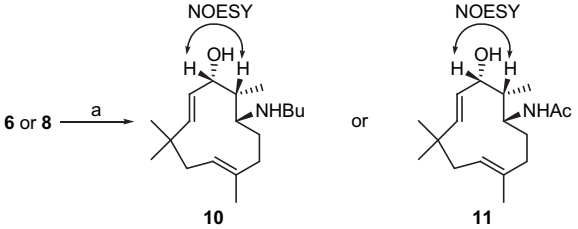
Epoxidation of the isolated double bond in zerumbone at C6 and C7 using *m*CPBA provided epoxide **12** in 97% yield [13]. This formation has high regioselectivity due to the high electron density and the less steric double bond. The epoxide **12** was further treated with an excess amount of various amines at room temperature providing corresponding amines **13**–**15**. As in the reaction of zerumbone, conjugate additions occurred at the C3 position and no nucleophilic addition of the remaining double bond (C10) was observed. As in a previous report, these amino derivatives were obtained as relatively *trans* configuration on C2 and C3. The relative orientation of the amino groups and epoxide oxygen showed that amination occurred on the same face of the epoxide. The reason for this observation was the steric repulsion of the two methyl groups at C2 and C6 of **12** which are located on one face of the ring while the oxide oxygen lies on the opposite face [14]. Further acetylation of **13** using Ac_2O in pyridine gave corresponding amide **16** in 66% yield (Scheme 4).

In contrast to the reaction of **1** with various amines, the excess KCN reacted with zerumbone at 40 °C for 3 days provided conjugate addition of cyanide ion at both C3 and C10 double bonds [15]. A mixture of four diastereoisomeric dicyano derivatives **17** was obtained. It was reported that in the major diastereoisomer (**17a**), two cyano groups were located on the same face of the ring while the two methyl groups at C2 and C6 lie on the opposite face (Scheme 5) [15].

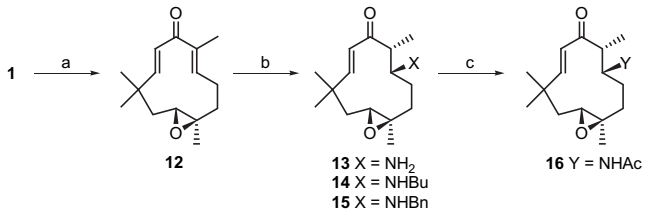
After treatment of **1** with dimethylamine in the presence of acetonitrile at room temperature, followed by stirring with excess KCN at 15 °C, the nitrile derivative **20** was detected [13]. This can be explained as that conjugate addition of dimethylamine at C3 gave intermediate **18**, while conjugate addition of the cyanide ion at C10 yielded intermediate **19**. After the easy elimination of the dimethylamino group, cyano **20** was observed as a sole product (Scheme 6). The ^1H NMR spectrum showed an olefinic proton at δ 5.95–6.50 (H3) and a singlet signal at δ 1.79 (CH_3-12) which indicates the presence of α,β-unsaturated ketone at C2 and C3. Two doublet signals of olefinic protons at H10 and H11 disappeared.

2.2. Biological activity [17]

Zerumbone (**1**) and its derivatives (**2**–**17** and **20**) were tested for their cytotoxicity against CCA cell lines; KKU-100, KKU-M139,



Scheme 3. Reagents and conditions: (a) $NaBH_4$, MeOH, 0 °C, 3 h, 81% (**10**), 54% (**11**).



Scheme 4. Reagents and conditions: (a) *m*CPBA, EtOAc, rt, 24 h, 97%; (b) NH_3 or $BuNH_2$ or $BnNH_2$, MeCN, rt, 5 days, 51% (**13**), 15% (**14**), 18% (**15**); (c) Ac_2O, pyridine, 0 °C, 10 h, 66%.

KKU-M156, KKU-M213 and KKU-M214 and their activities are shown in Table 1. Among these cell lines, KKU-100 was the least sensitive cell line which showed the highest IC_{50} value of 25.21 μM to ellipticine. It was found that zerumbone and alcohol derivatives showed no cytotoxic activity against all cell lines. Fortunately, primary amine **5** exhibited cytotoxic activity against KKU-100 cell line with an IC_{50} value of 16.44 μM which is about 1.5-fold higher toxicity than the ellipticine standard (IC_{50} = 25.21 μM). This compound also showed cytotoxicity against KKU-M139, KKU-M156, KKU-M213 and KKU-M214 cell lines with IC_{50} values ranging from 51 to 69 μM. Butylamine **6** possessed cytotoxicity against KKU-M213 and KKU-M214 cell lines with IC_{50} values of 36.61 and 41.55 μM, respectively. In contrast, benzylamine **7** showed no cytotoxicity against these five cell lines. It is interesting to note that the more polar primary amine **5** may play an important role in cytotoxicity.

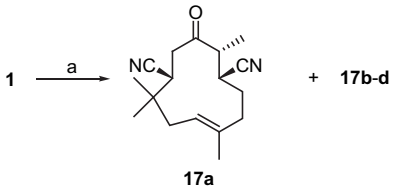
Zerumbol (**2**) showed no cytotoxicity against all CCA cell lines. But, when hydroxyamine **10** was synthesized and evaluated for cytotoxicity, it was found that this derivative displayed interesting results by showing cytotoxic activity against all cell lines with IC_{50} values ranging from 16 to 35 μM.

Epoxide **12** demonstrated weak cytotoxicity to KKU-M214 cell line with an IC_{50} value of 52.61 μM. From the cytotoxicity results of amine **5** and epoxide **12**, we hoped that epoxyamine **13** would exhibit good results on cytotoxicity testing. However, this compound displayed strong cytotoxicity only to the KKU-M214 cell line with an IC_{50} value of 18.30 μM. Epoxyamine **14** displayed good results to KKU-100 cell line by showing an IC_{50} value of 16.52 μM. In addition, this compound exhibited activity with IC_{50} values of 48.20, 37.63 and 51.00 μM against KKU-M156, KKU-M213 and KKU-M214, respectively.

Compound **20** showed strong cytotoxicity against the least sensitive cell line (KKU-100) with an IC_{50} value of 25.72 μM which is nearly equal to the ellipticine standard. This derivative also displayed moderate cytotoxicity against the other cell lines with IC_{50} values ranging from 37 to 56 μM. It was suggested that the nitrile group at the C10 position was favorable for CCA cell lines. All results show convincingly that amine, hydroxylamine, epoxyamine and nitrile groups are essential for cytotoxicity.

2.3. Molecular modeling simulation

From the results of the bioactivity testing, **5** containing an amine group exhibits the most potent antiproliferative activity, especially against KKU-100 cell line. In order to understand the modes of action of **5**, molecular modeling based on the docking approach was used to find possible targets of cholangiocarcinoma activity. Excessive activity of cyclin-dependent kinases (CDKs) has been observed as one of the mechanisms underlying pathological hyperproliferation [18]. Consequently, inhibition of CDKs could be a therapeutic option for treatment of cancer [19]. In addition, since the epidermal growth factor receptor (EGFR) is found to play an important role in cell cycle progression, cell proliferation and induction of apoptosis, it has become an important target in cancer therapy [20]. In cholangiocarcinoma cells, the expression of CDK-2, CDK-5 and EGFR in tumor tissues were found in both Thai intrahepatic cholangiocarcinoma (ICCs) and Japanese ICCs. Several reports indicate that the EGFR is frequently overexpressed in CCA [21]. Therefore, inhibition of CDK-2, CDK-5 or EGFR could provide control of the cell proliferation of certain cancers. Moreover, the expression of GSK-3β which plays an important in regulation of tumor cell proliferation through the activation of the Wnt/β-catenin pathway was found in 16/20 (80%) of Thai ICCs and in 16/20 (80%) of Japanese ICCs [22]. Therefore, in the course of our molecular modeling, we selected CDK-2, CDK-5, EGFR and GSK-3 for the elucidation of the possible characteristics of zerumbone derivatives in cholangiocarcinoma cells. The template of each protein target was constructed and validated by redocking with the native ligand. The chemical structures of these ligands are shown in Table 2 which summarizes the data and source of these structures [23–26]. The 3D-configuration of four inhibitors obtained from redocking with the developed protein templates were compared with the crystallographic poses. The results showed a good match of the docked and the crystallographic binding orientation with RMSD of less than 2.0 Å (Table 3). The validation results show that the constructed template is a good model system for predicting ligand binding orientation and binding affinity.



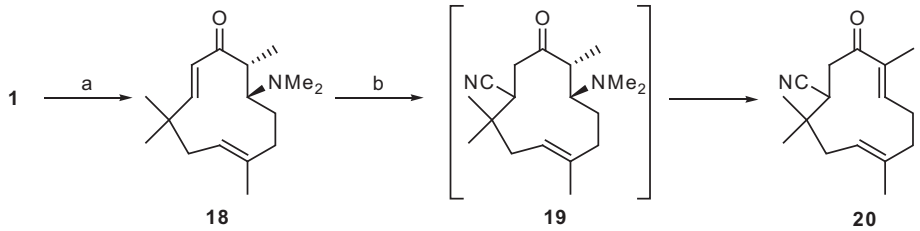
Scheme 5. Reagents and conditions: (a) excess KCN, MeCN-H_2O, 40 °C, 3 days, 42% (**17a**).

The docking results using AutoDock 4 indicate that **5** interacted with CDK-2 through the 2 H-bond at Asp145 with binding energy of −6.18 kcal/mol. In contrast, for CDK-5 and GSK-3 complexes it is seen that hydrogen bonds were not formed (Table 4).

The best binding interactions of **5** was formed in the complex with EGFR with the lowest binding energy of −7.24 kcal/mol. The binding energy of zerumbone, which showed no cytotoxic activity against KKU-100 at 75 μM, with EGFR was also calculated. The docking result showed that the binding energy of zerumbone was higher than the standard known inhibitor with the binding energy of −6.22 kcal/mol, as a result, the affinity of zerumbone decrease. Thus, zerumbone showed no antiproliferation activity at 75 μM. These patterns correlated with antipoliferative activities. For the binding mode of compound **5**, the core structure, sesquiterpene, is tightly bound to the pocket binding site with a hydrophobic interaction. The oxygen atom of the carbonyl group in **5** forms a hydrogen bond with the H–N of Lys721 (bond length 1.9 Å). The interaction between the primary amine and the oxygen atom of the Asp831 residue has a 1.7 Å bond length (Fig. 1). The docking result suggests that **5** might inhibit the proliferation of cancer via EGFR inhibition.

3. Conclusion

A series of zerumbone derivatives were synthesized and their in vitro cytotoxicity against cholangiocarcinoma cell lines were evaluated. Compounds **5**, **10**, **14** and **20** exhibited cytotoxic activity against all CCA cell lines to different extents, indicating their broad spectrum of anti-CCA effects. Our results indicate that these five CCA cell lines, with different histological types, respond differently to different compounds. This may be due to the variation in functional groups present in the compounds. The chemical structure



Scheme 6. Reagents and conditions: (a) Me_2NH, MeCN, rt, 5 days; (b) KCN, MeCN-H_2O, 15 °C, 2 days, 30% in two steps.

diversity of the four compounds reflects the biological activities. The presence of amine (**5**), hydroxylamine (**10**), epoxyamine (**14**), and nitrile (**20**) groups is believed to play an important role in potent anticancer activities. Among the tested compounds, **5,** which contained an amine group, exhibited higher potency. The docking result indicates that **5** may inhibit the proliferation of cancer through EGFR inhibition. This preliminary investigation is a good basis for further medicinal chemistry study.

4. Experimental

4.1. General

NMR spectra were recorded on a Varian Mercury plus spectrometer operating at 400 MHz (^{1}H) and at 100 MHz (^{13}C). IR spectra were recorded as KBr disks or thin films, using Perkin Elmer Spectrum One FT-IR spectrophotometer. Mass spectra were determined on Micromass Q-TOF 2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer with a Z-spray ES source (Micromass, Manchester, UK). Melting points were determined on a SANYO Gallenkamp melting point apparatus and were uncorrected. Thin layer chromatography (TLC) was carried out on MERCK silica gel 60 F_{254} TLC aluminum sheet. Column chromatography was done with silica gel 0.063–0.200 mm or less than 0.063 mm. Preparative layer chromatography (PLC) was carried out on glass supported silica gel plates using silica gel 60 PF_{254} for preparative layer chromatography. All solvents were routinely distilled prior to use.

4.2. Extraction and purification of zerumbone (*1*)

Dried rhizomes of *Z. zerumbet* Smith (4.0 kg) were ground into powder and then extracted with EtOAc (3 × 5 L) at room temperature. Removal of solvent under reduced pressure gave crude EtOAc extract (32.8 g) which was further subjected to column chromatography (CC) on silica gel 60 (500 g) and subsequently eluted with three solvents (hexane, EtOAc and MeOH) by gradually increasing polarity of elution solvents system. The eluents were collected and monitored by TLC resulting in 15 fractions (F_1–F_{15}). The solid in F_6 was separated by filtration, recrystallized from CH_2Cl_2-hexane to afford zerumbone (**1**, 15.0 g).

4.3. Preparation of zerumbone derivatives

4.3.1. Preparation of zerumbol (*2*)

To a solution of compound **1** (503 mg, 2.30 mmol) in dry THF (5.0 mL) was added $LiAlH_4$ (348 mg, 9.2 mmol) at 0 °C and the solution was stirred for 1 h. The reaction mixture was quenched with 10% HCl (10 mL) and extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification of crude oil by column chromatography (silica gel, 10% EtOAc:hexane) gave a white solid of compound **2**.

4.3.2. (±)-(2E,6E,10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trienol (*2*)

Yield 447 mg, 88%; mp 72–73 °C; IR ν_{max} 3290, 2955, 1445, 1297, 1076, 969 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.56 (1H, dd, J = 16.1, 7.2 Hz, H-11), 5.25 (1H, d, J = 16.1 Hz, H-10), 5.22 (1H, t, J = 7.5 Hz, H-3), 4.83 (1H, dd, J = 10.1, 4.0 Hz, H-7), 4.63 (1H, d, J = 7.2 Hz, H-1), 2.16–2.24 (1H, m, H-4), 2.04–2.14 (3H, m, H-4 and 2H-5), 2.01 (1H, dd, J = 13.7, 2.8 Hz, H-8), 1.80 (1H, dd, J = 13.7, 3.8 Hz, H-8), 1.67 (3H, s, CH_3-12), 1.44 (3H, s, CH_3-13), 1.08 (3H, s, CH_3-14 or 15), 1.06 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 141.8 (C2), 139.3 (C10), 133.0 (C6), 131.3 (C11), 124.9 (C7), 124.8 (C3), 78.6 (C1), 41.9 (C8), 39.1 (C5), 37.1 (C9), 29.4 (CH_3-14 or 15), 24.2 (CH_3-15 or 14), 22.9 (C4), 15.0 (CH_3-13), 12.7 (CH_3-12); HRMS *m/z* calcd mass for $C_{15}H_{24}O$ 243.1725 $[M + Na]^+$, found 243.1725.

4.3.3. Preparation of compound *3*

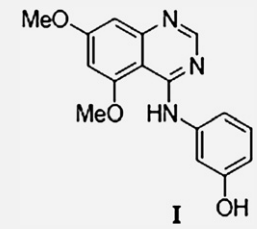
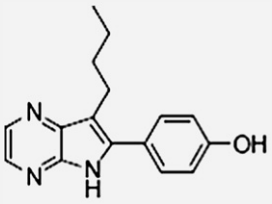
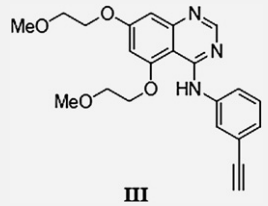
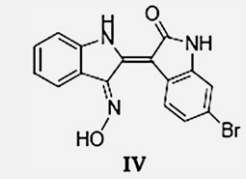
Compound **2** (35 mg, 0.16 mmol) was refluxed with acetic anhydride (1.0 ml) in the presence of pyridine (1.0 ml) for 30 min. The water was added and the mixture was extracted with CH_2Cl_2 (3

Table 1
Cytotoxicity of all compounds against cholangiocarcinoma cell lines.[a]

Compound	IC_{50}(μM)				
	KKU-100	KKU-M139	KKU-M156	KKU-M213	KKU-M214
Zerumbone	NR	NR	NR	NR	NR
5	16.44 ± 0.59	63.26 ± 5.48	69.04 ± 5.40	51.88 ± 2.25	59.65 ± 7.39
6	NR	NR	NR	36.61 ± 0.65	41.55 ± 0.58
10	35.33 ± 5.89	19.63 ± 1.26	26.34 ± 4.0	27.19 ± 1.29	16.05 ± 6.06
12	NR	NR	NR	NR	52.61 ± 3.88
13	NR	NR	NR	NR	18.30 ± 0.39
14	16.52 ± 0.84	NR	48.20 ± 1.85	37.63 ± 2.4	51.00 ± 0.78
17a–d	NR	NR	NR	NR	66.52 ± 3.63
20	25.72 ± 2.60	55.88 ± 4.4	37.30 ± 3.58	45.12 ± 0.89	54.50 ± 1.5
Other	NR	NR	NR	NR	NR
Ellipticine	25.21 ± 0.2	4.5 ± 0.28	9.34 ± 1.66	1.62 ± 0.08	1.01 ± 0.02

NR = no response at > 75 μM.
[a] Data shown are from triplicate experiments.

Table 2
Crystal ligand structures used in this study.

Ligand	PDB	Resolution Å
 I	1DI8	2.20
 II	1UNG	2.30
 III	1M17	2.60
 IV	1UV5	2.80

times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (40% CH_2Cl_2:Hexane) to give a white solid of compound **3**.

*4.3.4. (±)-(2E,6E,10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trienyl acetate (**3**)*

Yield 31 mg, 74%; mp 70–71 °C; IR ν_{max} 2955, 1737, 1368, 1240 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.54 (1H, dd, J = 15.8, 7.4 Hz, H-11), 5.48 (1H, d, J = 7.4 Hz, H-1), 5.31 (1H, d, J = 15.8 Hz, H-10), 5.26 (1H, t, J = 7.7 Hz, H-3), 4.84 (1H, dd, J = 10.6, 4.2 Hz, H-7), 2.12–2.20 (3H, m, 2H-4 and H-8), 2.02–2.10 (2H, m, H-5), 2.06 (3H, s, CH_3 of OAc), 1.78–1.84 (1H, m, H-8), 1.64 (3H, s, CH_3-12), 1.45 (3H, s, CH_3-13), 1.08 (3H, s, CH_3-14 or 15), 1.07 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 170.4 (C=O of OAc), 140.8 (C2), 138.7 (C6), 133.2 (C11), 127.7 (C7), 127.1 (C10), 124.7 (C3), 79.9 (C1), 41.1 (C8), 39.2 (C5), 37.3 (C9), 29.5 (CH_3-14 or 15), 23.8 (CH_3-15 or 14), 23.0 (C4), 21.2 (CH_3 of OAc), 15.0 (CH_3-13), 13.2 (CH_3-12); HRMS *m/z* calcd mass for $C_{17}H_{26}O_2$ 285.1825 $[M + Na]^+$, found 285.1835.

Table 3
Docking of crystal inhibitors.

Ligand	Macromolecule	Members in the highest cluster	$E_{binding}$ (kcal/mol)	RMSD from crystal orientation (Å)
I	CDK-2	87	−7.32	0.71
II	CDK-5	93	−6.53	0.92
III	EGFR	67	−7.69	1.67
IV	GSK-3	150	−9.14	0.56

Table 4
Geometric filters for training set of compound **5.**

Kinase targets	Number of H-bond	Geometric filters			$E_{binding}$ (kcal/mol)
		Amino acid residue	H-bonding atom	Distance (Å)	
CDK-2	2	Asp145	O (C=O)	2.211	−6.18
		Asp145	N (NH_2)	2.161	
CDK-5	0	–	–	–	−6.06
EGFR	2	Lys721	O (C=O)	1.951	−7.24
		Asp831	N (NH_2)	1.718	
GSK-3	0	–	–	–	−5.48

*4.3.5. Preparation of compound **4***

To a solution of compound **2** (39 mg, 0.18 mmol) in EtOAc (0.5 ml) was added *m*CPBA (37.4 mg, 0.22 mmol) at 0 °C. The solution was stirred for 1 h and then allowed to warm at room temperature for 24 h. The water was added and the mixture was extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (25% EtOAc:hexane) to give a white solid of **4**.

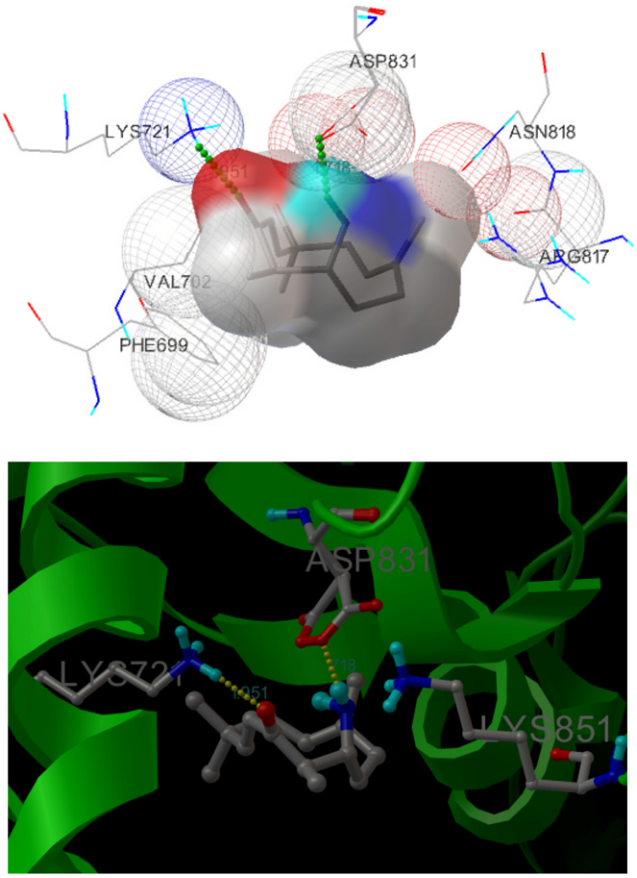


Fig. 1. Binding orientation of compound **5** on EGFR kinase.

*4.3.6. (±)-[6E,10E]-2,3-epoxy-2,6,9,9-tetramethylcycloundeca-6,10-dienol (**4**)*

Yield 41 mg, 15%; mp 84–85 °C; IR ν_{max} 3418, 2959, 1388, 1268, 1059 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.30 (1H, dd, J = 16.0, 6.9 Hz, H-11), 5.23 (1H, d, J = 16.0 Hz, H-10), 4.95 (1H, dd, J = 10.7, 3.7 Hz, H-7), 3.57 (1H, d, J = 7.5 Hz, H-1), 2.46 (1H, dd, J = 9.9, 4.3 Hz, H-3), 2.20–2.32 (2H, m, H-4 and H-5), 2.00–2.12 (2H, m, H-5 and H-8), 1.82 (1H, br d, J = 13.7 Hz, H-8), 1.55 (3H, s, CH_3-13), 1.36–1.44 (1H, m, H-4), 1.32 (3H, s, CH_3-12), 1.12 (3H, s, CH_3-14 or 15), 1.08 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 142.7 (C10), 132.0 (C6), 125.7 (C7), 125.2 (C11), 81.6 (C1), 65.6 (C2), 60.5 (C3), 40.2 (C8), 36.4 (C9), 36.1 (C5), 30.9 (CH_3-14 or 15), 29.8 (CH_3-15 or 14), 24.5 (C4), 15.0 (CH_3-13), 10.6 (CH_3-12); HRMS m/z calcd mass for $C_{15}H_{24}O_2$ 237.1849 $[M + H]^+$, found 237.1848.

*4.3.7. Preparation of amines **5**, **6** and **7***

A mixture of compound **1** (50 mg, 0.23 mmol) in MeCN (1.0 mL) and ammonia (30% in water, 2.0 mL) was stirred for 5 days at room temperature. The solution was concentrated on a rotary evaporator under reduced pressure. The water was added and the mixture was extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (10% MeOH:EtOAc) to give a white solid of compound **5**. The reaction of compound **1** with *n*-butylamine or benzylamine (excess, 4.0 mL) gave a yellow oil of **6** and a white solid of **7**, respectively.

*4.3.8. (±)-[6E,10E]-3-amino-2,6,9,9-tetramethylcycloundeca-6,10-dienone (**5**)*

Yield 32 mg, 56%; mp 90–91 °C; IR ν_{max} 3373, 3304, 1677, 1625, 1451, 995 cm^{-1}; ^1H NMR (CD_3OD) δ 6.13 (1H, d, J = 16.1 Hz, H-10), 6.08 (1H, d, J = 16.1 Hz, H-11), 5.04 (1H, dd, J = 11.7, 4.4 Hz, H-7), 3.23–3.35 (1H, m, H-3), 2.65 (1H, dq, J = 6.6, 2.9 Hz, H-2), 2.20 (1H, t, J = 12.4 Hz, H-8), 1.98–2.06 (1H, m, H-5), 1.78–1.90 (2H, m, H-5 and H-8), 1.30 (3H, s, CH_3-13), 1.10 (3H, s, CH_3-14 or 15), 1.08 (3H, s, CH_3-15 or 14), 0.98–1.02 (2H, m, H-4), 0.90 (3H, d, J = 6.6 Hz, CH_3-12); ^{13}C NMR (CD_3OD) δ 202.6 (C1), 152.5 (C10), 137.7 (C6), 127.8 (C11), 122.2 (C7), 54.4 (C2), 52.7 (C3), 41.2 (C8), 39.9 (C9), 37.7 (C5), 29.9 (C4), 28.7 (CH_3-14 or 15), 22.7 (CH_3-15 or 14), 16.2 (CH_3-13), 5.7 (CH_3-12).

*4.3.9. (±)-[6E,10E]-3-butylamino-2,6,9,9-tetramethylcycloundeca-6,10-dienone (**6**)*

Yield 62 mg, 93%; IR ν_{max} 3332, 1693, 1674, 1626, 1455, 998 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.12 (1H, d, J = 16.1 Hz, H-10), 5.58 (1H, d, J = 16.1 Hz, H-11), 5.00 (1H, dd, J = 11.7, 4.6 Hz, H-7), 2.97–2.99 (1H, m, H-3), 2.84–2.92 (1H, m, H-2), 2.60–2.66 (1H, m, H-1′), 2.47–2.55 (1H, m, H-1′), 2.12 (1H, t, J = 12.3 Hz, H-8), 1.95–2.02 (1H, m, H-5), 1.78–1.81 (2H, m, H-5 and H-8), 1.40–1.50 (2H, m, H-2′), 1.25–1.33 (2H, m, H-3′), 1.28 (3H, s, CH_3-13), 1.10 (3H, s, CH_3-14 or 15), 1.08 (3H, s, CH_3-15 or 14), 0.90–1.00 (2H, m, H-4), 0.85 (3H, t, J = 7.3 Hz, H-4′), 0.80 (3H, d, J = 6.6 Hz, CH_3-12); ^{13}C NMR ($CDCl_3$) δ 203.2 (C1), 152.0 (C10), 138.1 (C6), 127.7 (C11), 122.0 (C7), 58.5 (C3), 48.6 (C2), 46.8 (C1′), 41.3 (C8), 40.0 (C9), 38.3 (C5), 31.9 (C2′), 28.8 (CH_3-14 or 15), 28.7 (C4), 22.7 (CH_3-15 or 14), 20.4 (C3′), 16.2 (CH_3-13), 13.9 (C4′), 5.9 (CH_3-12).

*4.3.10. (±)-[6E,10E]-3-benzylamino-2,6,9,9-tetramethylcycloundeca-6,10-dienone (**7**)*

Yield 51 mg, 68%; mp 80–82 °C; IR ν_{max} 3421, 2922, 1687, 1624, 1042, 744, 701 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.26–7.60 (5H, m, Ar-H), 6.05 (1H, d, J = 16.1 Hz, H-10), 5.65 (1H, d, J = 16.1 Hz, H-11), 4.54 (1H, dd, J = 11.8, 4.2 Hz, H-7), 4.02 (1H, d, J = 13.6 Hz, H-1′), 3.77 (1H, d, J = 13.6 Hz, H-1′), 3.00 (1H, dq, J = 6.6, 2.7 Hz, H-2), 2.92 (1H, dd, J = 7.5, 2.7 Hz, H-3), 2.10 (1H, t, J = 12.3 Hz, H-8), 1.92–1.99 (1H, m, H-5), 1.61 (NH), 1.75 (1H, t, J = 12.0 Hz, H-5), 1.68 (1H, dd, J = 12.0, 4.5 Hz, H-8), 1.31 (3H, s, CH_3-13), 1.08 (3H, s, CH_3-14 or 15), 1.01–1.05 (1H, m, H-4), 0.96 (3H, d, J = 6.6 Hz, CH_3-12), 0.86–0.94 (1H, m, H-4), 0.77 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 203.1 (C1), 151.4 (C10), 140.0 (C-Ar), 137.9 (C-Ar), 137.9 (C6), 128.6 (C-Ar), 128.4 (C-Ar), 127.9 (C11), 127.3 (C-Ar), 121.9 (C7), 56.0 (C3), 50.5 (C1′), 47.4 (C2), 41.3 (C8), 39.7 (C9), 37.5 (C5), 29.0 (CH_3-14 or 15), 28.9 (C4), 22.7 (CH_3-15 or 14), 16.4 (CH_3-13), 6.0 (CH_3-12); HRMS m/z calcd mass for $C_{22}H_{31}NO$ 348.2303 $[M + Na]^+$, found 348.2303.

*4.3.11. Preparation of compounds **8** and **9***

Compound **5** (or **6**) (0.34 mmol) reacted with acetic anhydride (1.0 mL) in the presence of pyridine (1.0 mL) at 0 °C for 8 h. The water was added and the mixture was extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (50% EtOAc:hexane) to give a white solid of **8** or a yellow solid of **9**, respectively.

*4.3.12. (±)-[6E,10E]-3-(N-Acetylamino)-2,6,9,9-tetramethylcycloundeca-6,10-dienone (**8**)*

Yield 92 mg, 92%; mp 199–200 °C; IR ν_{max} 3300, 1694, 1633, 1547 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.40 (1H, d, J = 16.2 Hz, H-11), 6.14 (1H, d, J = 16.2 Hz, H-10), 5.15 (1H, dd, J = 11.7, 4.2 Hz, H-7), 4.47 (1H, br d, J = 8.3 Hz, H-3), 2.90 (1H, dq, J = 6.7, 2.6 Hz, H-2), 2.20 (1H, t, J = 12.3 Hz, H-8), 1.98 (3H, s, CH_3 of NHAc), 1.90–1.96 (1H, m, H-5), 1.85 (1H, dd, J = 13.0, 4.4 Hz, H-8), 1.70 (1H, t, J = 12.3 Hz, H-5), 1.36 (3H, s, CH_3-13), 1.19 (3H, s, CH_3-14 or 15), 1.13 (3H, s, CH_3-15 or 14), 0.95–1.00 (2H, m, H-4), 0.95 (3H, d, J = 6.7 Hz, CH_3-12); ^{13}C NMR ($CDCl_3$) δ 202.6 (C1), 170.7 (C=O of NHAc), 152.1 (C10), 136.5 (C6), 128.4 (C11), 123.2 (C7), 50.8 (C2), 50.7 (C3), 41.5 (C8), 40.0 (C9), 37.0 (C5), 28.8 (CH_3-14 or 15), 27.4 (C4), 22.9 (CH_3 of NHAc), 22.8 (CH_3-15 or 14), 16.3 (CH_3-13), 6.6 (CH_3-12); HRMS m/z calcd mass for $C_{17}H_{27}NO_2$ 300.1939 $[M + Na]^+$, found 300.1939.

*4.3.13. (±)-[6E,10E]-3-(N-Acetylbutylamino)-2,6,9,9-tetramethylcycloundeca-6,10-dienone (**9**)*

Yield 97 mg, 85%; mp 72–73 °C; IR ν_{max} 3554, 3476, 1693, 1619 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.62 (1H, d, J = 16.2 Hz, H-11), 6.16 (1H, d, J = 16.2 Hz, H-10), 5.14–5.20 (2H, m, H-3 and H-7), 3.28–3.60 (1H, m, H-1′), 3.14–3.23 (1H, m, H-1′), 2.80 (1H, dq, J = 6.7, 2.2 Hz, H-2), 2.22–2.32 (1H, m, H-8), 2.18 (3H, s, CH_3 of NHAc), 1.95 (1H, dd, J = 12.5, 6.5 Hz, H-5), 1.87 (1H, dd, J = 13.2, 4.3 Hz, H-8), 1.56–1.66 (2H, m, H-4 and H-5), 1.46–1.56 (2H, m, H2′), 1.41 (3H, s, CH_3-13), 1.28–1.36 (2H, tq, J = 14.7, 7.3 Hz, H3′), 1.26 (3H, s, CH_3-14 or 15), 1.15 (3H, s, CH_3-15 or 14), 0.99 (3H, d, J = 6.7 Hz, CH_3-12), 0.94 (3H, t, J = 7.3 Hz, H-4′), 0.80–0.90 (1H, m, H-4); ^{13}C NMR ($CDCl_3$) δ 202.5 (C1), 171.8 (C=O of NHAc), 151.7 (C10), 136.2 (C6), 128.8 (C11), 123.7 (C7), 54.1 (C3), 51.7 (C2), 45.9 (C1′), 41.7 (C8), 40.2 (C9), 37.1 (C5), 33.3 (C2′), 29.0 (CH_3-14 or 15), 23.1 (CH_3-15 or 14), 23.0 (C4), 22.3 (CH_3 of NHAc), 20.2 (C3′), 16.3 (CH_3-13), 13.7 (C4′), 8.3 (CH_3-12); HRMS m/z calcd mass for $C_{21}H_{35}NO_2$ 356.2565 $[M + Na]^+$, found 356.2566.

*4.3.14. Preparation of compounds **10** and **11***

To a solution of compound **6** (or **8**) (0.10 mmol) in MeOH (1.0 mL) was added $NaBH_4$ (0.15 mmol) at 0 °C and the solution was stirred for 3 h. The reaction mixture was quenched with 10% HCl (10 mL) and extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (100% EtOAc) to give a white solid of compound **10** (or **11**).

*4.3.15. (±)-[6E,10E]-3-butylamino-2,6,9,9-tetramethylcycloundeca-6,10-dienol (**10**)*

Yield 81%; IR ν_{max} 3428, 2959, 1563, 1413, 1022 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.26 (1H, br d, J = 15.3 Hz, H-10), 4.98–5.10 (2H, m, H-7 and H-11), 3.98 (1H, br s, H-1), 2.58–2.70 (1H, m, H-3), 2.40–2.52 (2H, m, H-1′), 2.02–2.12 (1H, m, H-8), 1.80–1.94 (3H, m, H-2 and 2H-5), 1.68 (1H, t, J = 13.0 Hz, H-8), 1.54 (3H, s, CH_3-13), 1.38–1.46 (2H, m, H-2′), 1.20–1.30 (4H, m, 2H-4 and 2H-3′), 1.00 (6H, s, 2CH_3-14, 15), 0.88 (3H, d, J = 6.9 Hz, CH_3-12), 0.84 (3H, t, J = 7.2 Hz, H-4′); ^{13}C NMR ($CDCl_3$) δ 145.1 (C10), 140.2 (C6), 128.8 (C11), 128.4 (C7), 79.9 (C1), 60.2 (C3), 50.8 (C2), 44.4 (C1′), 44.1 (C8), 42.1 (C9), 39.0 (C5), 34.7 (C4), 31.9 (CH_3-14 or 15), 29.4 (CH_3-15 or 14), 27.8 (C2′), 24.3 (C3′), 23.0 (CH_3-13), 17.6 (C4′), 11.7 (CH_3-12); HRMS *m/z* calcd mass for $C_{19}H_{35}NO$ 294.2797 $[M + H]^+$, found 294.2798.

*4.3.16. (±)-[6E,10E]-3-(N-Acetylamino)-2,6,9,9-tetramethylcycloundeca-6,10-dienol (**11**)*

Yield 54%; mp 84–85 °C; IR ν_{max} 3383, 1632, 1290, 1023, 978 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.23–5.36 (2H, m, H-10 and H-11), 5.13 (1H, d, J = 8.0 Hz, H-7), 4.19–4.23 (1H, m, H-1), 2.88–2.91 (1H, m, H-3), 1.90–2.02 (2H, m, H-2 and H-8), 1.89 (3H, s, CH_3 of NHAc), 1.77–1.83 (1H, m, H-8), 1.58–1.63 (2H, m, H-5), 1.57 (3H, s, CH_3-13), 1.11–1.28 (2H, m, H-4), 1.12 (3H, s, CH_3-14 or 15), 1.03 (3H, s, CH_3-15 or 14), 0.91 (3H, d, J = 7.3 Hz, CH_3-12); ^{13}C NMR ($CDCl_3$) δ 170.4 (C=O of NHAc), 138.9 (C10), 136.2 (C6), 126.4 (C11), 124.1 (C7), 74.7 (C1), 48.1 (C3), 42.9 (C8), 41.3 (C9), 38.2 (C2), 35.4 (C5), 27.8 (CH_3-14, 15), 23.0 (C4), 22.9 (CH_3 of NHAc), 18.0 (CH_3-13), 8.9 (CH_3-12); HRMS *m/z* calcd mass for $C_{17}H_{29}NO_2$ 302.2096 $[M + Na]^+$, found 302.2096.

*4.3.17. Preparation of compound **12***

To a solution of **1** (39 mg, 0.18 mmol) in EtOAc (0.5 mL) was added *m*CPBA (37.4 mg, 0.22 mmol) at 0 °C, the solution was stirred for 1 h and then allowed to warm at room temperature for 24 h. The water was added and the mixture was extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (20% EtOAc:hexane) to give a crytalline solid of compound **12**.

*4.3.18. (±)-[2E,10E]-6,7-epoxy-2,6,9,9-tetramethylcycloundeca-2,10-dienone (**12**)*

Yield 41 mg, 97%; mp 84–85 °C; IR ν_{max} 2963, 1656, 1262, 1119, 971 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.02–6.10 (3H, m, H-3, H-10 and H-11), 2.70 (1H, dd, J = 11.2, 1.5 Hz, H-7), 2.34–2.45 (2H, m, H-4), 2.21–2.27 (1H, m, H-5), 1.89 (1H, d, J = 14.0 Hz, H-8), 1.80 (3H, s, CH_3-12), 1.41 (1H, dd, J = 14.0, 11.2 Hz, H-8), 1.26–1.34 (1H, m, H-5), 1.25 (3H, s, CH_3-14 or 15), 1.18 (3H, s, CH_3-13), 1.03 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 202.8 (C1), 159.4 (C10), 147.7 (C3), 139.4 (C2), 128.2 (C11), 62.7 (C7), 61.3 (C6), 42.6 (C8), 38.1 (C5), 35.9 (C9), 29.7 (CH_3-14 or 15), 24.6 (C4), 24.0 (CH_3-15 or 14), 15.6 (CH_3-13), 12.0 (CH_3-12); HRMS *m/z* calcd mass for $C_{15}H_{22}O_2$ 257.1517 $[M + Na]^+$, found 257.1517.

*4.3.19. Preparation of compounds **13**–**15***

The synthesis of compounds **13**–**15** was similar as described in the preparation of compounds **5**–**7**.

*4.3.20. (±)-[10E]-3-amino-6,7-epoxy-2,6,9,9-tetramethylcycloundeca-10-enone (**13**)*

Yield 51%; mp 126–127 °C; IR ν_{max} 3382, 1693, 1631, 1009, 914 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.29 (1H, d, J = 16.0 Hz, H-11), 6.20 (1H, d, J = 16.0 Hz, H-10), 3.12–3.17 (1H, m, H-3), 2.62–2.68 (2H, m, H-2 and H-7), 2.00–2.06 (1H, m, H-5), 1.84 (1H, t, J = 12.5 Hz, H-8), 1.30 (1H, dd, J = 11.3, 2.5 Hz, H-8), 1.20 (3H, s, CH_3-14 or 15), 1.06 (3H, s, CH_3-15 or 14), 1.04 (3H, s, CH_3-13), 0.94–0.98 (3H, m, 2H-4 and H-5), 0.90 (3H, d, J = 6.7 Hz, CH_3-12); ^{13}C NMR ($CDCl_3$) δ 203.1 (C1), 150.8 (C10), 127.7 (C11), 62.0 (C6), 60.7 (C7), 54.3 (C3), 53.2 (C2), 40.2 (C8), 36.6 (C5), 36.1 (C9), 29.4 (CH_3-14 or 15), 27.4 (C4), 23.1 (CH_3-15 or 14), 16.6 (CH_3-13), 5.6 (CH_3-12); HRMS *m/z* calcd mass for $C_{15}H_{26}NO_2$ 252.1964 $[M + H]^+$, found 252.1962.

*4.3.21. (±)-[10E]-3-butylamino-6,7-epoxy-2,6,9,9-tetramethylcycloundeca-10-enone (**14**)*

Yield 15%; mp 76–77 °C; IR ν_{max} 3434, 1688, 1628, 1114, 917 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.30 (1H, d, J = 16.0 Hz, H-11), 6.25 (1H, d, J = 16.0 Hz, H-10), 2.92–3.00 (2H, m, H-2 and H-7), 2.66–2.74 (2H, m, H-3 and H-1′), 2.44–2.52 (1H, m, H-1′), 2.05–2.09 (1H, m, H-5), 1.90 (1H, d, J = 13.8 Hz, H-8), 1.42–1.50 (2H, m, H-2′), 1.34–1.41 (3H, m, H-8 and 2H-3′), 1.26 (3H, s, CH_3-14 or 15), 1.15 (3H, s, CH_3-15 or 14), 1.10 (3H, s, CH_3-13), 1.00–1.10 (2H, m, H-4), 1.00–1.05 (1H, m, H-5), 0.93 (3H, t, J = 7.2 Hz, H-4′), 0.90 (3H, d, J = 6.4 Hz, CH_3-12); ^{13}C NMR ($CDCl_3$) δ 203.8 (C1), 150.3 (C10), 127.8 (C11), 62.0 (C6), 60.7 (C7), 60.7 (C3), 47.8 (C2), 47.0 (C1′), 40.5 (C8), 37.2 (C5), 36.2 (C9), 32.4 (C2′), 29.6 (CH_3-14 or 15), 26.2 (C4), 23.3 (CH_3-15 or 14), 20.4 (C3′), 16.8 (CH_3-13), 14.0 (C4′), 5.8 (CH_3-12); HRMS *m/z* calcd mass for $C_{19}H_{34}NO_2$ 308.2590 $[M + H]^+$, found 308.2593.

*4.3.22. (±)-[10E]-3-benzylamino-6,7-epoxy-2,6,9,9-tetramethylcycloundec-10-enone (**15**)*

Yield 18%; mp 80–81 °C; IR ν_{max} 3350, 1686, 1626, 1113, 917, 749, 704 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.28–7.42 (5H, m, Ar-H), 6.16 (1H, d, J = 16.0 Hz, H-10), 5.84 (1H, d, J = 16.0 Hz, H-11), 4.02 (1H, d, J = 13.4 Hz, H-1′), 3.72 (1H, d, J = 13.4 Hz, H-1′), 3.00 (1H, dq, J = 6.6, 3.2 Hz, H-2), 2.80 (1H, dd, J = 9.3, 3.2 Hz, H-3), 2.14 (1H, dd, J = 11.2, 1.6 Hz, H-7), 2.00 (1H, dd, J = 8.6, 8.2 Hz, H-5), 1.74 (1H, d, J = 13.5 Hz, H-8), 1.20–1.28 (1H, m, H-8), 1.08–1.14 (2H, m, H-4), 1.06 (3H, s, CH_3-14 or 15), 1.04 (3H, s, CH_3-13), 0.96 (3H, d, J = 6.6 Hz, CH_3-12), 0.90 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 203.8 (C1), 150.1 (C10), 139.7 (C-Ar), 128.7 (C-Ar), 128.4 (C-Ar), 127.7 (C11), 127.5 (C-Ar), 61.7 (C6), 60.4 (C7), 57.2 (C3), 50.3 (C1′), 46.4 (C2), 40.7 (C8), 36.5 (C5), 36.0 (C9), 29.6 (CH_3-14 or 15), 25.9 (C4), 23.0 (CH_3-15 or 14), 16.8 (CH_3-13), 5.7 (CH_3-12); HRMS *m/z* calcd mass for $C_{22}H_{32}NO_2$ 342.2433 $[M + H]^+$, found 342.2433.

*4.3.23. Preparation of compound **16***

Acetylation reaction of compound **13** (23 mg 0.08 mmol) was similar as described above.

*4.3.24. (±)-[10E]-3-(N-Acetylamino)-6,7-epoxy-2,6,9,9-tetramethylcycloundeca-10-enone (**16**)*

Yield 15.6 mg, 66%; mp 205–206 °C; IR ν_{max} 3243, 3072, 1699, 1632, 1551, 1107, 916 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.59 (1H, d, J = 16.1 Hz, H-11), 6.29 (1H, d, J = 16.1 Hz, H-10), 4.47 (1H, dt, J = 7.5, 3.1 Hz, H-3), 2.96 (1H, dq, J = 6.7, 3.1 Hz, H-2), 2.85 (1H, dd, J = 11.2, 1.7 Hz, H-7), 1.98–2.03 (1H, m, H-5), 2.01 (3H, s, CH_3 of NHAc), 1.90–1.95 (1H, m, H-8), 1.36–1.41 (1H, dd, J = 11.2, 2.5 Hz, H-8), 1.34 (3H, s, CH_3-13), 1.19–1.28 (1H, m, H-4), 1.15 (3H, s, CH_3-14 or 15), 1.12 (3H, s, CH_3-15 or 14), 1.00–1.06 (1H, m, H-4), 0.95 (3H, d, J = 6.7 Hz, CH_3-12), 0.95–0.98 (1H, m, H-5); ^{13}C NMR ($CDCl_3$) δ 202.6 (C1), 170.1 (C=O of NHAc), 150.6 (C10), 128.4 (C11), 61.1 (C6), 60.3 (C7), 52.0 (C3), 49.7 (C2), 40.8 (C8), 36.3 (C9), 36.0 (C5), 29.5 (CH_3-14 or 15), 24.9 (C4), 23.4 (CH_3-13), 23.3 (CH_3 of NHAc), 16.8 (CH_3-15 or 14), 6.5 (CH_3-12); HRMS *m/z* calcd mass for $C_{17}H_{27}NO_3$ 316.1889 $[M + Na]^+$, found 316.1889.

*4.3.25. Preparation of compounds **17a-d***

To a solution of compound **1** (66 mg, 0.30 mmol) in MeCN (1.0 mL) was added the solution of KCN (63 mg, 0.97 mmol) in H_2O (1.0 mL), the reaction mixture was stirred for 3 days at 40 °C. The reaction mixture was quenched with H_2O (10 mL) and extracted

with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification of crude oil by PLC (30% EtOAc:Hexane) gave a white solid of a major diastereomer **17a**.

4.3.26. (±)-[6E]-3,10-dicyano-2,6,9,9-tetramethylcycloundeca-6-enone (**17a**)

Yield 35 mg, 42%; mp 115–116 °C; IR ν_{max} 2962, 2234, 1726 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.11 (1H, dd, J = 10.5, 4.0 Hz, H-7), 3.36 (1H, dd, J = 19.4, 5.2 Hz, H-11), 3.25–3.28 (1H, m, H-10), 2.83–2.86 (2H, m, H-2 and H-3), 2.50 (1H, dd, J = 19.4, 3.2 Hz, H-11), 2.12–2.22 (3H, m, 2H-8 and H-5), 1.84–1.92 (3H, m, 2H-4 and H-5), 1.45 (3H, s, CH_3-13), 1.26 (3H, s, CH_3-14 or 15), 1.22 (3H, d, J = 6.8 Hz, CH_3-12), 1.16 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 202.2 (C1), 136.1 (C6), 122.1 (C7), 121.9 (CN at C10), 121.6 (CN at C3), 45.9 (C3), 41.3 (C8), 41.1 (C11), 38.9 (C5), 35.9 (C9), 33.5 (C10), 33.3 (C2), 31.9 (CH_3-14 or 15), 24.5 (C4), 21.5 (CH_3-15 or 14), 16.3 (CH_3-13), 11.2 (CH_3-12); HRMS m/z calcd mass for $C_{17}H_{24}N_2O$ 295.1786 $[M + Na]^+$, found 295.1787.

4.3.27. Preparation of compound **20**

Dimethylamine derivative **18** was prepared in the same procedure as described above and used as the starting material for the further step. A mixture of **18** (50 mg, 0.19 mmol) and KCN (210.3 mg, 3.2 mmol) in MeCN:H_2O (1:0.5 mL) was stirred for 2 days at 15 °C. The solution was concentrated on a rotary evaporator under reduced pressure. The water was added and the mixture was extracted with EtOAc (3 times). The organic layer was combined, washed with water, brine and dried over anhydrous Na_2SO_4. Evaporation of solvent gave a crude oil, which was purified by PLC (50% EtOAc:hexane) to give a white solid of compound **20**.

4.3.28. [2E,6E]-10-cyano-2,6,9,9-tetramethylcycloundeca-2,6-dienone (**20**)

Yield 32 mg, 60% in the final step; mp 104–105 °C; IR ν_{max} 2966, 2232, 1672, 1461, 1267, 817 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.95–6.05 (1H, m, H-3), 5.11 (1H, t, J = 5.9 Hz, H-7), 2.88–3.07 (1H, br s, H-11), 2.72–2.80 (1H, m, H-10), 2.53–2.66 (1H, m, H-11), 2.42–2.52 (1H, m, H-5), 2.24–2.42 (3H, m, 2H-4 and H-5), 2.00–2.22 (2H, m, H-8), 1.79 (3H, s, CH_3-12), 1.57 (3H, s, CH_3-13), 1.19 (3H, s, CH_3-14 or 15), 1.07 (3H, s, CH_3-15 or 14); ^{13}C NMR ($CDCl_3$) δ 202.9 (C1), 143.7 (C3), 137.0 (C6), 134.9 (C2), 122.8 (C7), 120.2 (CN at C10), 39.9 (C8), 39.6 (C4), 39.0 (C11), 37.3 (C9), 36.6 (C10), 28.3 (CH_3-14 or 15), 25.8 (CH_3-15 or 14), 24.6 (C5), 15.8 (CH_3-13), 12.5 (CH_3-12); HRMS m/z calcd mass for $C_{16}H_{23}NO$ 268.1677 $[M + Na]^+$, found 268.1677.

4.4. Cytotoxicity assay

Cytotoxic activity of the compounds was determined by the sulforhodamine B (SRB) assay [17]. Briefly, 190 mL/well of cell suspensions (0.5–1.0 × 10^5 cells/mL) were seeded in 96-well plates and incubated at 37 °C for 24 h. Then 10 mL/well of each concentration of the compounds was added in triplicate to obtain final concentration of 0.025, 0.16, 0.8, 4 and 20 μg/well and 0.1% DMSO was used as the solvent-control wells. The plates were incubated for 1 h (starting cells) and 72 h at 37 °C. At the end of each exposure time, the medium was removed. The cells were fixed with 20% (w/v) trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) at 4 °C for 1 h and stained with 0.4% (w/v) SRB (Sigma) dissolved in 1% acetic acid (Sigma) at room temperature for 30 min. After five times washing with 1% acetic acid, protein-bound dye was solubilized with 10 mM Tris base, pH10 (Sigma) and the absorbance (OD) at 510 nm was determined with an ELISA plate reader (ELX-800; Bio-Tek Instruments, Inc.). Percentage cell viability was calculated as: [(OD treated cells on day 3–OD starting cells)/(OD control on day 3–OD starting cells)] × 100. Dose-response curves were plotted and growth inhibition of 50% (IC_{50}) was determined at compound concentration which results in 50% reduction of total protein increase in control cells.

4.5. Investigation the target protein of compounds **1** and **5** by docking study

4.5.1. Template preparation

A crystallographic structure of cyclin-dependent kinases 2 (CDK-2) [23], cyclin-dependent kinases 5 (CDK-5) [24], epidermal growth factor receptor (EGFR) [25] and glycogen synthase kinase 3 (GSK-3) [26] bound to the selective inhibitor 4-[3-hydroxyanilino]-6,7-dimethoxyquinazoline (ligand **I**) for CDK-2, 6-phenyl[5H]pyrrolo[2,3-b]pyrazine (ligand **II**) for CDK-5, [6,7-bis(2-methoxyethoxy)quinazoline-4-yl]-(3-ethynylphenyl)amine (ligand **III**) for EGFR and 6-bromoinidirubin-3′-oxime (ligand **IV**) for GSK-3 were selected as the docking template. These structures were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) (PDB codes: 1DI8 for CDK-2, 1UNG for CDK-5, 1M17 for EGFR and 1UV5 for GSK-3, Table 2). In order to prepare the target protein as a template, ligand and crystallographic water were removed. Hydrogens and Gasteiger charges were assigned by using AutoDockTools (ADT) [27]. Atomic salvation parameters, based on the Stouten model, and fragmental volumes were added in accordance with the AutoDock force field [28]. Cubic affinity grid maps for each atom type in the ligand set (plus an electrostatics map) centered on the cavity with dimensions of 45 × 45 × 45 Å and 0.375 Å spacing between grid points were computed using AutoGrid 4.0 for each protein model. Lennard–Jones parameters 12-10 and 12-6 were used for modeling H-bonds and Van Der Waals interactions, respectively.

4.5.2. Template validation

To validate the constructed templates, the crystal structures of native ligands including ligands **I**, **II**, **III** and **IV** were used to redock with its template, CDK-2, CDK-5, EGFR and GSK-3, respectively.

To prepare the inhibitors to dock to the constructed template, all hydrogens were added and Gasteiger charges were assigned for all inhibitors. ADT was used to merge nonpolar hydrogens and define which bonds were rotatable for each ligand.

AutoDock 4 was employed to perform the docking calculation. Each ligand was docked to the developed CDK-2, CDK-5, EGFR and GSK-3 templates by using a Lamarkian genetic algorithm search (LGA). Due to the stochastic nature of genetic search algorithms, each ligand was docked in 150 trials. Docking trial for each ligand was initiated with a randomly generated population of 150 ligand orientations and the highest affinity configuration was selected after 2 million energy evaluations had been performed. Standard AutoDock parameters were used for the genetic algorithm: 2% point mutation; 80% cross over rate; 6% local search rate.

The resulting ligand configurations, from 150 trials within a 2.0 RMSD (root-mean-square deviation) tolerance of each other, were grouped together in clusters. The results of the docking experiments were evaluated by calculating the positional RMSD of the corresponding atoms of each conformation. The final docked structure, RMSD from the bound crystal structure, docked energy and predicted free energy of binding were all used to analyze its interaction with the active site.

5. Molecular docking of 1 and 5 to the constructed protein templates

To prepare **1** and **5** for docking with four constructed templates, all hydrogens were added and Gasteiger charges were assigned

[29]. The ADT was used to merge nonpolar hydrogens and define which bonds were rotatable. **1** and **5** was docked to the four protein templates by using a Lamarkian genetic algorithm search. Due to the stochastic nature of genetic search algorithms, each ligand was docked in 150 trials. Each docking trial for ligand was initiated with a randomly generated population of 150 ligand orientations and the highest affinity orientation was selected after 2 million energy evaluations had been performed. Standard AutoDock parameters were used for the genetic algorithm: 2% point mutation; 80% cross over rate; 6% local search rate [28]. The resulting ligand orientations from 150 trials with a 2.0 Å RMSD tolerance of each other were grouped together as clusters. The final docked structure, docked energy and predicted free energy of binding were used for the analysis of its interaction with the active site.

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