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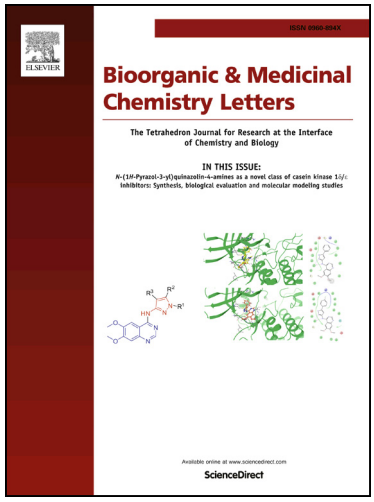

Synthesis of novel forskolin isoxazole derivatives with potent anti-cancer activity against breast cancer cell lines

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Synthesis of novel forskolin isoxazole derivatives with potent anti-cancer activity against breast cancer cell lines

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ABSTRACT

Forskolin C_1-isoxazole derivatives (3,5-regioisomers) (**11a-e, 14,15a-h** and **15,16a-g**) were synthesized regioselectively by adopting 1,3-dipolar cycloadditions. These derivatives were tested using estrogen receptor positive breast cancer cell lines MCF-7 and BT-474. Majority of the compounds exhibited activity against the p53-positive MCF-7 breast cancer cells but not against the p53-negative BT-474 breast cancer cells. Among forskolin derivatives, compounds **11a, 11c, 14a, 14f, 14g, 14h, 15b, 16g** and **17b** exhibited higher anti-cancer activity against MCF-7 cell line with an $IC_{50} \leq 1\mu M$. The derivative **14f** exhibited highest activity in both p53-positive (MCF-7) and p53-negative (BT-474) breast cancer cell lines with an IC_{50} of 0.5µM.

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Breast cancer is the most common cancer in the women worldwide. Natural products such as Docetaxel, Paclitaxel, Eribuline, Vinblastine and Doxorubicin were used for the treatment of breast cancer [1-4]. Importantly, diterpenoids such as Forskolin **(1)**, Fulvestrant **(2)**, Megestrol **(3)**, Formestane **(4)**, Atamestane **(5)** and Exemestane **(6)** were shown to have anti-cancer activity **(Figure-1)**, specifically against estrogen receptor-positive breast cancer [5-13].

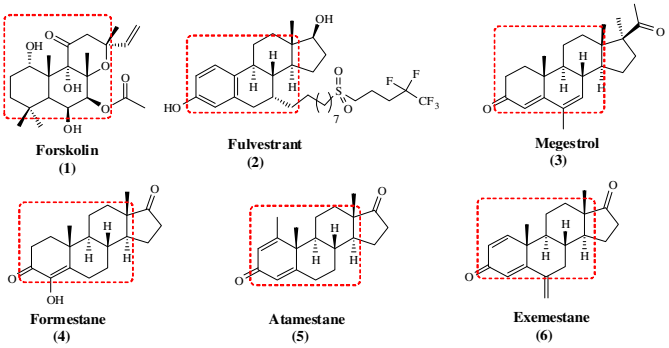

Figure-1: Some diterpenes as breast cancer agents.

Forskolin, a labdane diterpene natural product, has been isolated from the roots of an Indian sub-continent plant *Coleus forskohlii* [14]. Forskolin exhibits a wide range of pharmacological properties such as anti-obesity [15], Cardiovascular [16], asthma [17], glaucoma [18], antihypertensive, inotropic [19,20], anti-cancer activity [21,22], stimulates the

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adenylyl cyclase activity and increases intracellular levels of cyclic AMP [23]. It has been reported that the order of reactivity of the hydroxyls in forskolin is 1-OH > 6-OH > 9-OH. [24] Generally, Heterocyclic compounds improve the pharmacokinetic and pharmacodynamic properties of anti-cancer drugs by enhancing lipophilicity, polarity or other physicochemical features. In the present study, we thus introduced novel isoxazoles at C_1-OH and tested their activity on breast cancer cell lines.

1,3-dipolar cycloaddition is one of the best methods for the synthesis of 5-membered heterocyclic compounds. In this reaction, 1,3-dipole reacts with dipolarophiles in a symmetry allowed [π4s+π2s] manner. According to Sustmanns approach [25] these cycloadditions are of three types: HOMO-Controlled (Type-I: the interaction of the dipole HOMO with the dipolarophile LUMO is greatest), HOMO-LUMO Controlled (Type-II: both frontier orbital interactions are large) and LUMO-Controlled (Type-III: the interaction of the dipole LUMO with the dipolarophile HOMO is greatest). Earlier studies suggested that reactions involving aryl nitrile oxide 1,3-dipoles belong to Type-II molecular orbital interactions in FMO theory [26-28]**.** In nitrile oxide ($C\equiv N^+-O^-$) dipole, larger HOMO coefficient is on electronegative oxygen, while the larger LUMO coefficient is on the carbon. The HOMO of the 1,3-dipole when interacts with the LUMO of the dipolarophile favors the formation of one particular regioisomer, while the LUMO of 1,3-dipole when interacts with the HOMO of the dipolarophile favors the formation of an opposite regioisomer. Generally, isoxazole regioisomers are distinguished using ^{1}H NMR. In the 3,5-disubstituted isomer, isoxazole H-4 appears as a singlet in the range of δ 6.59-6.76 ppm, whereas in the 3,4-isomer, isoxazole H-5 appears downfield in the range of δ 7.50-8.50 ppm [29].

A small library of isoxazole substituted forskolin derivates was synthesized adopting 1,3-dipolar cycloadditions. To synthesize isoxazole as the sole product at C_1-OH, the double bond at C-14,15 of forskolin (**1**) was initially reduced with 10% Pd/C in MeOH resulting in the formation of 14,15-dihydroforskolin **(7)** **(Scheme 1: Step-1)**. This intermediate (**7**) was then propargylated at 1-OH in the presence of K_2CO_3 and NaI resulting in the formation of 1-O-propargyl-14,15-dihydroforskolin **(8)** and 1-O-propargyl-6-O-acetyl-7-deacetyl14,15-dihydroforskolin **(9)** regioisomers in 1:1 ratio **(Scheme 1: Step-2)**. 1,3-dipolar cycloadditions [30] were finally performed with aryl nitrile oxides (*in situ* generated from **10a-d, h)** on pure 1-O-propargyl-14,15-dihydroforskolin **(8)** resulting in the formation of the isoxazoles: 1-O-(3-(substituted phenyl)isoxazole-5-yl)methyl-14,15-dihydroforskolins (**11a-e**) **(Scheme 1: Step 3)** (ex. procedure [31]). Similarly, **(Scheme-1, step-2** and **3)**, propargylation of forskolin **(1)** gave two regioisomers 1-O-propargyl-forskolin **(12)** and 1-O-propargyl-6-O-acetyl-7-deacetyl-forskolin **(13)** **(Scheme-2, step-1)** [32]. Since, these compounds (**12** and **13**) have both terminal alkyne and alkene moieties, reaction with aryl nitrile oxides (*in situ* generated from **10a-h)** adopting 1,3-dipolar cycloaddition strategy furnished two products C_1-isoxazole and C_{13}-isoxazoline substituted C_1-isoxazole: **14a-h** & **15a-h** (in 3:1 ratio from

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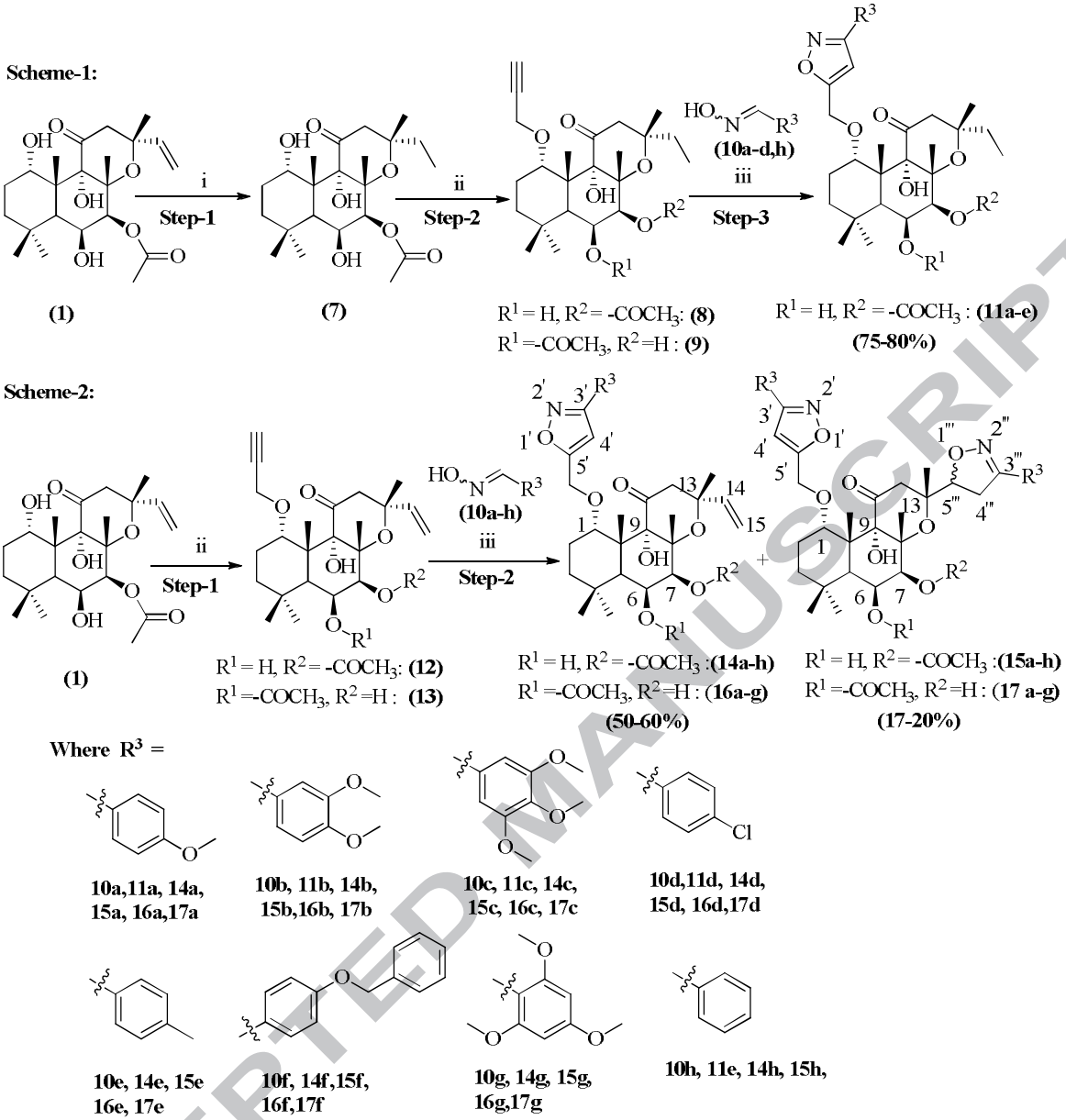

Regents and conditions: i) 10% Pd/ C,MeOH, H_2 gas, r.t, 1-2hr; ii) Propargyl bromide, K_2CO_3,NaI, acetone,reflux, 24hr; iii) 9-12% NaOCl, DCM, NEt_3, 0^0C-rt, 6hr.

compound **12**) and **16a-g** & **17a-g** (in 3:1 ratio from compound **13**) **(Scheme-2, step-2).**

All the novel forskolin derivatives were characterized from their ^{1}H NMR and/or ^{13}C NMR, IR and ESI-MS/HRMS-(ESI) spectra. In the ^{1}H NMR of **11a-e**, **14a-h, 15a-h, 16a-g** and **17a-g**, C_1-isoxazole proton appeared as singlet in the range of δ 6.43-6.91ppm indicating a proton at 4th position of isoxazole ring, confirming exclusive formation of 3,5-disubstituted isoxazole regioisomer. Among the protons of C-13 isoxazolines (**15a-h** and **17a-g**), H-5‴ appeared in the range of δ 4.89-6.17 (br. s) and two H-4‴ protons appeared in the range of δ 3.21-5.13 (m, 2H), indicating the formation of 3,5-disubstituted-4,5-dihydroisoxazoline exclusively. ESI-MS of **11a** molecular ion peak was observed at m/z 622 $[M+Na]^+$, HRMS (ESI) of molecular ion peak of **14a** was observed at m/z : 615.3289

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$[M+NH_4]^+$, **15a** was observed at : m/z 747.3491 $[M+H]^+$, **16a** was observed at: m/z 615.3288 $[M+NH_4]^+$ and **17a** was observed at : m/z 747.3495 $[M+H]^+$.

NOESY correlations for final isoxazole products **11a, 14a, 15a**, **16a** and **17a (See figure-2)** revealed that the isoxazole proton H-4′ showed nOe with aromatic protons H-2″ / H-6″ while $4''_a$-OCH_3 protons showed nOe with H-3″/H-5″ indicating that they are spatially closer. For compounds **11a, 14a** and **15a,** 6-OH proton showed nOe with 8-CH_3, H-5 proton showed nOe with 9-OH/1-OCH_2, and 9-OH proton showed nOe with H-$12_{(a)}$ indicating that these protons are at the axial position. Further, 6-$OCOCH_3$ protons of **16a** and **17a** showed nOe with H-2_a / $4_{(a)}$-CH_3 / 10-CH_3 and H-2_a respectively indicating the presence of 6-acetyl group at axial position, while 7-$OCOCH_3$ of **11a, 14a** and **15a** protons showed no nOe with other protons indicating the presence of 7-acetyl group at equatorial position [32]. For compounds **15a** and **17a,** nOe was observed between 1) H-$12_{(a)}$ proton and H-14 2) H-15 proton and H-2‴″/H-6‴″, and 3) 4‴″a-OCH_3 protons and H-3‴″/H-5‴″ (for NOESY spectra see supporting information).

Aryl nitrile oxides which were used in above reactions **(Scheme-1 & Scheme-2)** were generated *in situ* from substituted benzaldehyde oximes (**10a-h**), in the presence of NaOCl as oxidant and triethylamine as the base. **10a-h** were synthesized from substituted benzaldehydes in the presence of hydroxylamine hydrochloride and sodium acetate

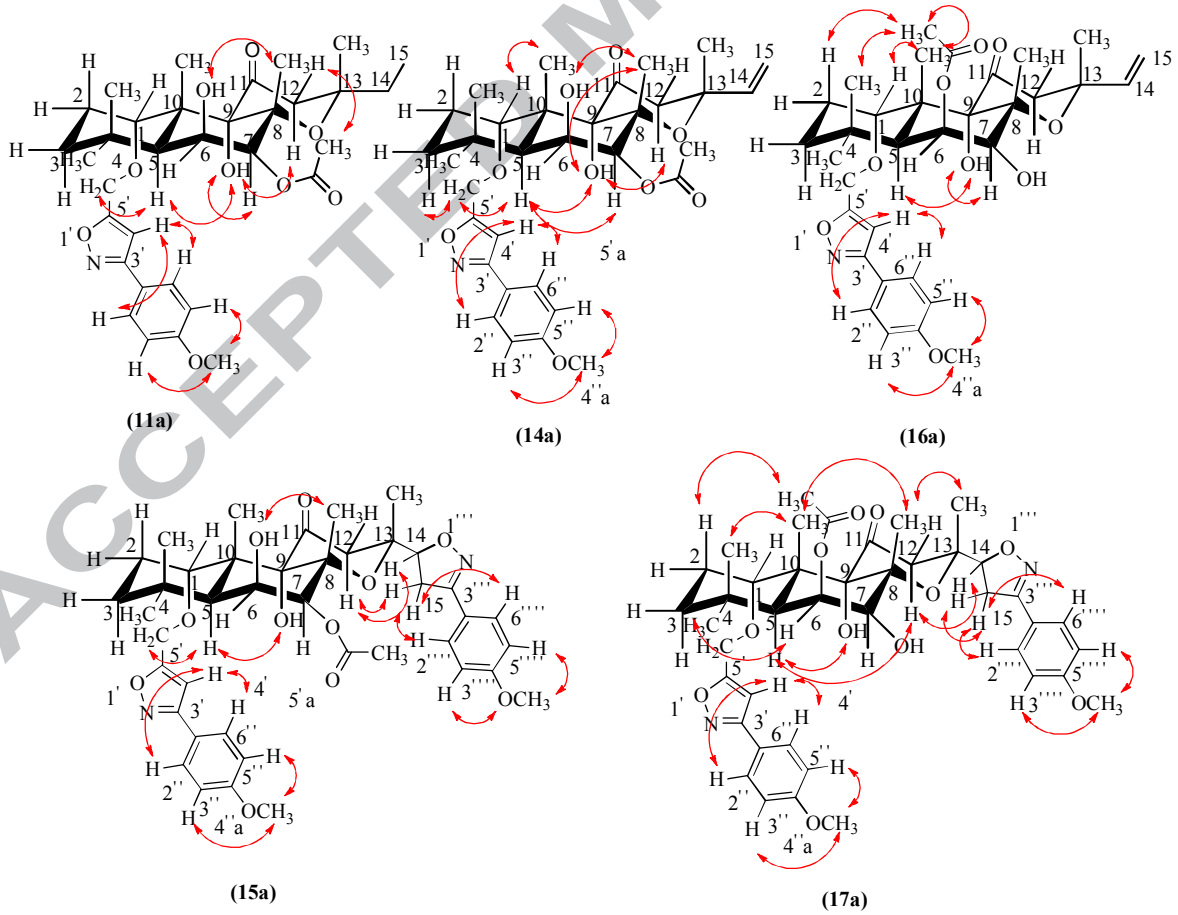

Figure-2: NOESY Correlations of 11a, 14a, 15a, 16a and 17a

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were heated at reflux in ethanol for 1hour to afford oximes **(10a-h)** with excellent yields (80-96%) [29].

Forskolin was previously shown to have activity against breast cancer cell lines [5]. Furthermore, the anti-proliferative activity of forskolin was shown to be mediated by the tumor suppressor protein p53 [6]. Thus, we tested the anti-cancer activity of forskolin derivatives on both p53-positive MCF-7 and p53-negative BT-474 cell lines [33]. The parental forskolin displayed cellular IC_{50} of 63.3±5.7 µM against MCF-7 but lacked any anti-cancer activity against the BT-474 cell line (IC_{50} > 100 µM) **(Table 1)**. Of the 32 derivatives tested, 24 compounds showed more than 2-fold higher anti-cancer activity than the parental forskolin against MCF-7 cell line, of which 18 derivatives showed more than 10-fold activity **(Table 1)**. However, only 10 derivatives displayed more than 2-fold anti-cancer activity against BT-474 cell line, of which 8 derivatives displayed more than 10-fold activity **(Table 1)**. Notably, 9 forskolin derivatives displayed very high anti-cancer activity at sub micro-molar concentrations ($IC_{50} \leq 1$ µM) against the p53-positive MCF-7 cell line. Of these, the compound **14f** (1-O-(3-(4-benzyloxyphenyl-isoxazole-5-yl)methyl-forskolin) with an acetyl group at 7th position exhibited the highest activity in both the MCF-7 and BT-474 cell lines with an IC_{50} of 0.5µM **(Table 1)**. On the contrary, the compound **16f** (1-O-(3-(4-benzyloxyphenyl-isoxazole-5-yl)methyl-6-O-acetyl-7deacetyl-forskolin) with an acetyl group at 6th position lacked anti-cancer activity with cellular IC_{50} values

Table-1: Anti cancer activity of forskolin derivatives

	Anti-cancer activity (IC_{50} in µM)				
Compound	**MCF-7**	**BT-474**	**Compound**	**MCF-7**	**BT-474**
Forskolin	63.3±5.7	>100	**15f**	52.6±4.6	79.6±18.7
8	10±0	>100	**14g**	0.5±0	>100
11a	0.5±0	2.1±0.1	**15g**	*	*
11b	9.6±2	>100	**14h**	0.5±0	5±1
11c	0.5±0	29.3±10.6	**15h**	35.3±10	>100
11d	1.6±0.2	7±2	**13**	30±0	>100
11e	2.6±1.1	9.1±0.2	**16a**	57.3±9.4	>100
-	-	-	**17a**	79.3±14.4	>100
12	>100	>100	**16b**	12±2.6	28.3±12.3
14a	0.5±0	4±1	**17b**	0.5±0	>100
15a	3±0.8	4.3±1.1	**16c**	4.4±0.3	>100
14b	1.1±0.4	>100	**17c**	*	*
15b	0.5±0	>100	**16d**	4±1	>100
14c	55.3±9.2	>100	**17d**	*	*
15c	*	*	**16e**	32±7.5	>100
14d	5.6±1.1	>100	**17e**	59±14.7	>100
15d	*	*	**16f**	84.6±15.5	>100
14e	7±2.6	75±22.9	**17f**	3.3±0.5	1.3±0.5
15e	5.6±2	>100	**16g**	0.5±0	>100
14f	0.5±0	0.5±0	**17g**	*	*

* = formed in very less amounts

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greater than that of the parental forskolin **(Table 1)**. Additionally, compounds **11a, 14a, 14f, 14h, 15a** and **17f** displayed anti-cancer activity in both cell lines with cellular $IC_{50} \leq 5$ μM **(Table 1)**. Further analysis of these four compounds **11a, 14a, 14f** and **14h** showed significant cytotoxicity than that of the parental forskolin **(Figure 3)** [34].

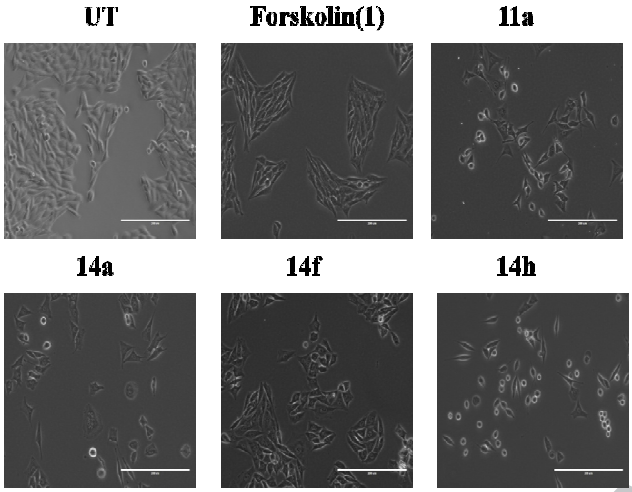

Figure-3: Anti cancer activity of UT (untreated), Forskolin (1), **11a, 14a, 14f** and **14h** in MCF-7 cell line.

In conclusion, we describe the synthesis of 3,5-disubstituted isoxazole derivatives of forskolin **(11a-e, 14a-h, 15a-h, 16a-g** and **17a-g)** with good yields in a regioselective manner by Nitrile Oxide Alkyne/alkene Cycloadditions (NOAC). Interestingly, propargyl alkyl ether having dipolarophiles with nitrile oxides as 1,3-dipoles in the 1,3-dipolar cycloadditions (Dipole LUMO controlled-Type-II**:** dipole LUMO and dipolarophile HOMO interactions are favored) gives exclusively 3,5-disubstituted-regioisomers only. This synthesis is in agreement with earlier studies [28, 30] which indicates that electron rich dipolarophiles react with nitrile oxides as 1,3-dipole in 1,3-dipolar cycloadditions strategy. Furthermore, compounds **11a, 11c, 14a, 14f, 14g, 14h, 15b, 16g** and **17b** exhibited significant anti-cancer activity in p53-positive MCF-7 cell line, while compounds **14f** and **17f** showed anti-tumor activity in p53-negative BT-474 cell line.

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Supplementary data

Supplementary data provided with this article as a separate file.

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31. *General method for the Synthesis of 1-O-(3-substitutedphenyl)*isoxazol*e-5-yl)-methyl-14,15-dihydroforskolins* **(11a-e).** To a stirred solution of propargylated forskolins **(8, 12** and **13)** (0.22mmol) in 5ml dichloromethane, add triethylamine (0.22mmol) and 2ml of aqueous NaOCl (9-12%) at 0^0C, add corresponding benzaldehyde oximes **(10a-h)** (0.22mmol) slowly in a portion of 30min time in each case. Reaction was stirred at room temperature for 6-8hr. After completion of the reaction, the mixture was dissolved in 20ml of water and extract with DCM. Separate the organic layer, dried over anhydrous Na_2SO_4, concentrated under reduced pressure, purified by column chromatography (EtOAc/Hexane), 60-120 mesh silica gel to afford pure compounds **(11a-e, 14a-h** and **15a-h** (~3:1 ratio**), 16a-g** and **17a-g** (~3:1 ratio)**)** as solids. *1-(3-(4-Methoxyphenyl)isoxazole-5-yl)methoxy)-14,15-dihydroforskolin **(11a)**.* Off white solid, m.p-120-122^{0}C, yield : 85%; IR (KBr) (cm^{-1}): 3498(OH), 3334(OH), 2926(CH), 2854 (CH), 1731(OCO), 1708(CO), 1608 (C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 8.8 Hz, 2H, H-2″, 6″), 6.97 (d, *J* = 8.8Hz, 2H, 3″, 5″), 6.45 (s,1H, H-4′ (isoxazole)), 6.44 (s, 1H, 9-OH), 5.52 (d, *J* = 4.2Hz, 1H, H-7), 4.67 (d, *J* =13.0Hz, 1H, OCH_2), 4.48–4.40 (m, 2H, OCH_2, H-6), 4.36 (br.s, 1H, H-1), 3.86 (s, 3H, OCH_3), 3.27 (d, *J* =15.5Hz, 1H, H-12_e), 2.28-2.22 (m, 1H,H-12_a), 2.14 (s, 3H, $COCH_3$), 2.17– 2.12 (m, 1H, H-2_a), 2.10 – 2.03 (m, 1H, H-5), 1.83 (s, 1H, 6-OH), 1.75 – 1.63 (m, 3H, H-14, H-2_e), 1.60 (s, 3H, 8-CH_3), 1.50 (s, 3H, 10-CH_3), 1.53 – 1.41 (m, 1H, H-3_a), 1.26 (s, 3H, 4-$CH_{3(e)}$), 1.20 (s, 3H,13-CH_3), 1.17–1.11(m,1H, H-3_e), 1.02 (s, 3H, 4-$CH_{3(a)}$), 0.89 (dd, *J* = 8.8, 6.1Hz, 3H, H-15). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 209.50 (C=O), 169.57 (OCO), 167.90 (C-3′), 162.04 (C-4″), 161.08 (C-5′), 128.25(C-2″,6″), 121.26 (C-1″),114.34 (C-3″, 5″), 101.21 (C-4′), 84.25 (C-13), 82.41 (C-8), 81.21 (C-1), 76.91 (C-9), 76.25 (C-7), 69.90 (C-6), 61.54 (OCH_2), 55.35 (4′a-OCH_3), 49.52 (C-12), 44.00 (C-5), 43.78(C-10), 38.55(C-3), 36.43(C-4), 34.18 (C-14), 32.78(4_e-CH_3), 29.46(8-CH_3), 24.04 (13-CH_3), 23.82 (4a-CH_3), 21.11(C-2), 20.70 ($OCOCH_3$), 20.17 (10-CH_3), 7.85 (C-15). ESI-MS: m/z 622 $[M+Na]^+$.

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33. **Cell viability assay:** Breast cancer (MCF7 and BT474) cell lines were used to test the anti-cancer activity. $2x10^3$ cells per well were cultured in triplicates in a 96-well plate in the presence of novel compounds. Untreated cells were taken as the control. 48 hours following the treatment with indicated compounds,

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alamar blue was added and cell viability was analyzed by assaying samples at 570 nm wavelength. Percentage inhibition of cell viability upon treatment with drugs when compared to that of the untreated cells was calculated and IC_{50} values were derived and shown as mean $\pm$ SD in **Table-1**.

34. **Microscopy:** MCF7 cells were plated in 6-well plates and were treated with indicated drugs. Untreated well was taken as control. 48 hours after drug treatment, cells were observed under microscope for morphological changes and photographs were taken and shown in **Figure-3.**

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Graphical Abstract

Synthesis of novel forskolin isoxazole derivatives with potent anti-cancer activity against breast cancer cell lines

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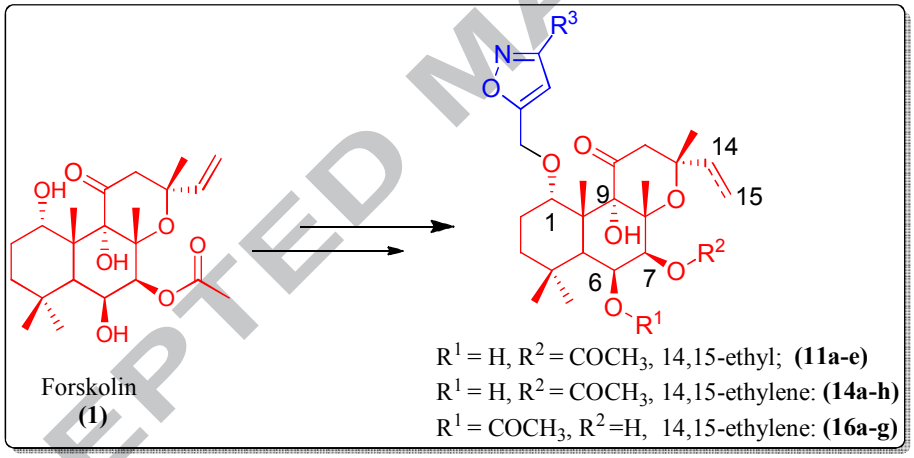

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