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PAPER

Polymer supported synthesis of novel benzoxazole linked benzimidazoles under microwave conditions: *In vitro* evaluation of VEGFR-3 kinase inhibition activity†

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An efficient soluble polymer-supported method has been developed for the parallel synthesis of substituted benzimidazole linked benzoxazoles using focused microwave irradiation. The key step involves the amidation of 4-hydroxy-3-nitrobenzoic acid with polymer-immobilized *o*-phenylenediamine. Application of mild acidic conditions promoted the ring closure to furnish the benzimidazole ring. After hydrogenation of the nitro-group to amine, the resulted polymer conjugates underwent efficient ring closure with various alkyl, aryl and heteroaryl isothiocyanates to generate the polymer-bound benzimidazolyl benzoxazoles. The polymer-bound compounds were finally cleaved from the support to furnish benzimidazole linked benzoxazole derivatives. The efficacy of the resultant angular bis-heterocyclic library was studied against vascular endothelial growth factor receptor (VEGFR-3). The preliminary screening of these novel compounds exhibits moderate to high inhibition (IC_{50} = 0.56–1.42 μM). This protocol provides an easy access to novel angular bis-heterocycles which have potential for the discovery of novel leads for targeted cancer therapeutics.

Introduction

Among many diseases, cancer is still a major cause of death in the world and remains a major focus for ongoing research and development. In recent years a promising approach to the therapeutic intervention of cancer has focused on antiangiogenesis therapies.[1] This approach takes advantage of the idea of inhibiting the blood supply to tumors to deplete the oxygen and nutrients and subsequently arrest tumor cell growth as well as proliferation. This approach has been found to be effective and there are presently several antiangiogenic drugs undergoing various stages of evaluation in clinical trials.[2] Recently, two small molecule inhibitors sunitinib (**A**) and sorafenib (**B**) have been approved, in addition to AMG 706 (**C**), as antiangiogenic drugs[3] (Fig. 1). In spite of these achievements, it remains an important challenge to develop new drugs in order to overcome drug resistance and maintain the steady progress in cancer research. Hence the discovery of novel lead compounds for drug development plays an important role in cancer drug research. Concomitantly, coupled with high throughput screening, development of effective methodologies for rapid synthesis of diversified libraries of small heterocyclic molecules is of great importance.[4]

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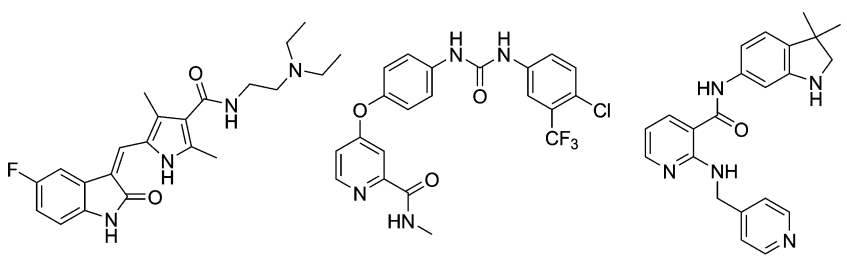
† Electronic supplementary information (ESI) available: General experimental procedures and ^{1}H NMR, ^{13}C NMR, crude HPLC, LRMS, HRMS and FT-IR spectral data of compounds **15a–p**. CCDC reference numbers 783608. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c0ob00547a



Sunitinib (A) **Sorafenib (B)** **AMG 706 (C)**

Fig. 1 Examples of small molecule kinase inhibitors.

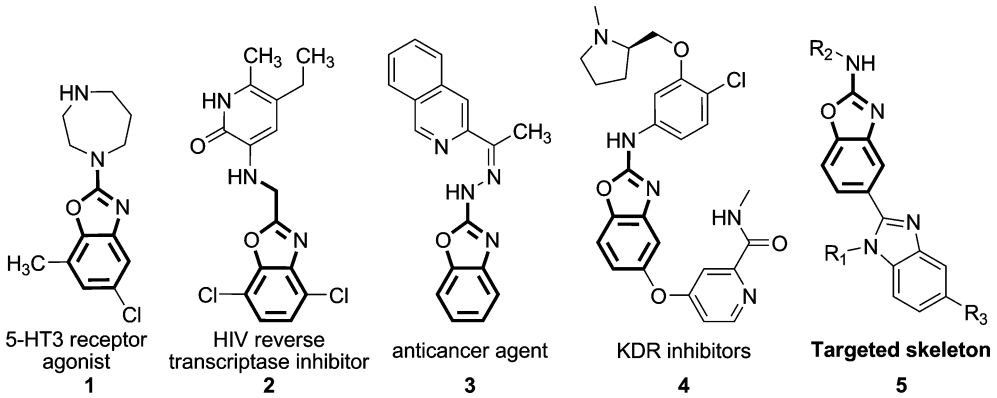


Fig. 2 Biologically relevant benzoxazole derivatives and the targeted skeleton.

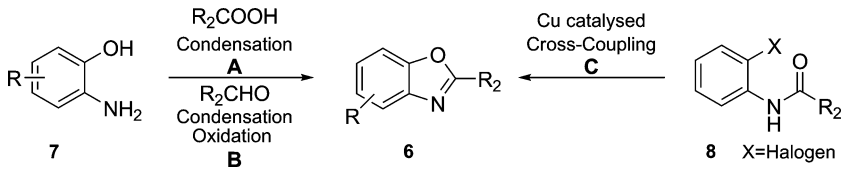


Fig. 3 Reported methods to prepare benzoxazole derivatives.

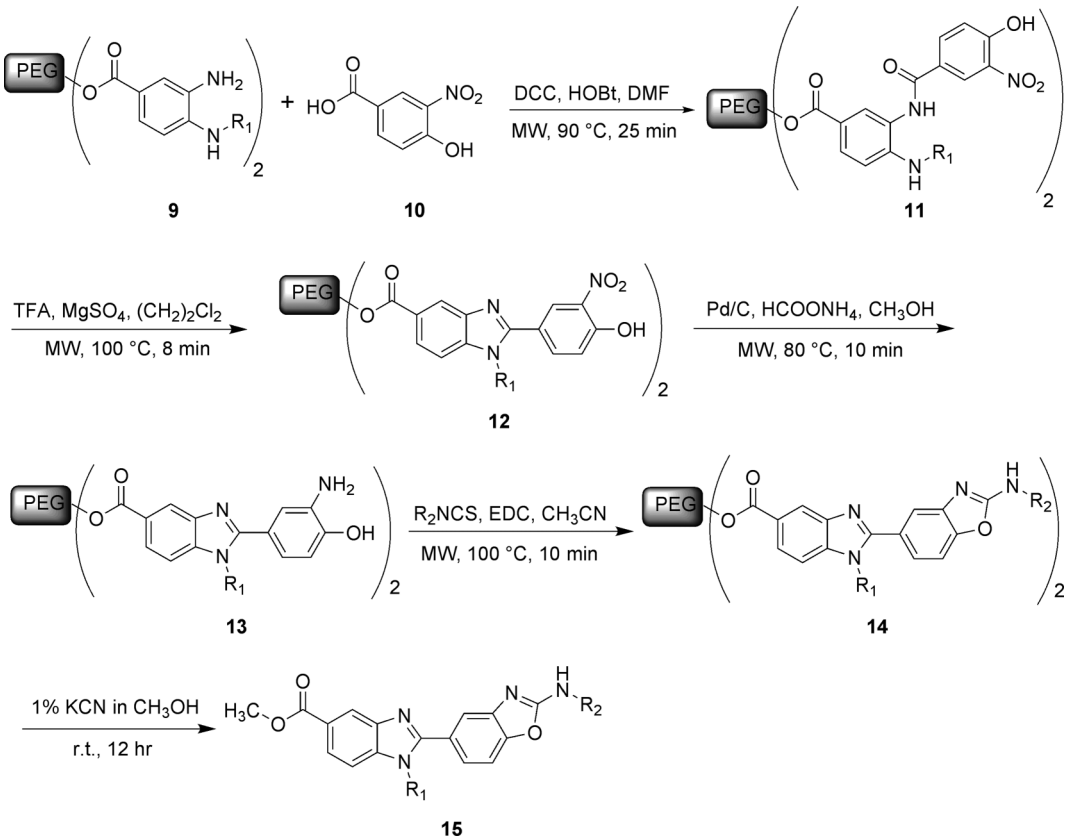
Motivated by these synthetic challenges, chemists have developed various solid phase as well as solution phase synthetic methods for the construction of important heterocycles.[5] Due to the drawbacks such as heterogeneous reaction conditions, nonlinear kinetics, solvation problems, and difficulty in monitoring reactions that solid-phase synthesis experiences, chemists have sought alternative methodologies such as soluble polymer-supported synthesis which can be conducted in homogeneous organic media. Additionally, realizing that organic reactions and reaction sequences involved in synthesizing the target molecules should preferably be facile, swift and efficient, there has been a shift in emphasis towards the use of microwave irradiation in recent years.[6] Microwave-assisted organic synthesis has received constant attention because the organic reactions can now be performed in sealed vessels in a temperature and pressure controlled mode, providing reproducible results for producing biologically interesting small heterocyclic molecules. In our previous studies, we reported the application of microwave techniques in multi-step liquid phase synthesis to generate heterocyclic libraries.[7]

Although the medicinal chemistry fraternity has put up a brave front to cover a wide range of structurally and functionally derivatized heterocyclic compounds, the libraries of bis-heterocyclic compounds have not been extensively studied so far. The linked bis-heterocyclic scaffolds with two or more heteroatom-containing rings have significance to afford additional binding opportunities as well as improved diversification scope, and hence have been recognized as the most potent heterocyclic motifs for the development of novel therapeutics.[8] Particularly, benzimidazoles[9] and benzoxazoles[10] are privileged heterocycles known for a wide range of biological activities (Fig. 2). Prominently, the benzoxazole derivatives have been found to be 5-HT_3 receptor agonists[11] **1**, HIV reverse transcriptase inhibitors[12] L-697,661 **2** and anticancer agents[13] NSC-693638 **3**. Similarly, the benzoxazole ring is an intriguing heterocycle embedded in a novel class of potent KDR inhibitors **4**.[14] In an interest to develop biologically promising bis-heterocyclic molecules, our research focused on developing a novel benzoxazole linked benzimidazole skeleton **5** and further extension of this study to biologically evaluate these molecules for VEGFR-3 kinase inhibition.

A literature survey revealed that only a few methods were reported for the synthesis of benzoxazole derivatives which involve the condensation of 2-aminophenol with either carboxylic acids or aldehydes under high temperature (Fig. 3A and 3B).[15] Another method involving metal-catalyzed cross-coupling is also known (Fig. 3C).[16] However, all these methods suffered from harsh reaction conditions, longer reaction times and limited diversification. Prompted by the necessity to develop an easy and efficient method to construct benzoxazole derivatives and our interest in the benzimidazole linked benzoxazole scaffold, here we disclose the multidisciplinary synergetic approach comprising polymer-supported synthesis with microwave synthesis to generate a bis-heterocyclic skeleton-containing small library and its *in vitro* evaluation of VEGFR-3 kinase inhibition activity.

Results and discussion

The present strategy commenced with the synthesis of polymer-immobilized *ortho*-phenylenediamine **9** from 4-fluoro-3-nitrobenzoic acid with built-in structural diversity (R_1) through a three-step protocol (Scheme 1).[17] In an effort to obtain the target molecule, compound **9** was *N*-acylated at the primary amine functionality with 4-hydroxy-3-nitrobenzoic acid **10** *via* DCC activation. Accordingly, anilide conjugates **11** were obtained by the condensation of acid **10** with PEG conjugates **9** through the activated ester generated by DCC and HOBT in DMF in 12 h reflux. However, the application of microwave irradiation (90 °C) reduced the reaction time to 25 min. For the construction of the benzimidazole ring, anilide conjugates **11** were subjected to acid catalyzed cyclisation in the presence of



Scheme 1 General strategy of microwave-assisted synthesis of benzimidazolyl benzoxazoles.

10% trifluoroacetic acid and $MgSO_4$ in 1,2-dichloroethane. The formation of the conjugate **12** was achieved in 10 h under reflux conditions.

In order to achieve the target compound quickly, we applied the microwave irradiation under sealed vessel conditions (100 °C, 8 bar) at this stage, which further reduced the reaction time to 8 min. The polymer conjugate **12** was purified by precipitating out the reaction mixtures with excess cold ether. For the reduction of the nitro group, conjugate **12** was treated with ammonium formate and 10% palladium on activated charcoal in methanol. Formation of the amine conjugates **13** was achieved under reflux conditions for 2 h. However, by the application of microwave irradiation at 80 °C, the desired conjugates **13** were obtained within 10 min. It is noteworthy to say that the metal reduction proceeded smoothly under microwave conditions. After completion, palladium and excess ammonium formate were removed by filtration. Amine conjugates **13** were obtained in pure form by further precipitation in cold ether.

Our main goal was the construction of the benzoxazole ring together with introduction of additional sets of diversity. It has been realized that the elaboration of intermediate **13** to the desired core structure required one carbon electrophile. In an effort to build the benzoxazole motif to mimic the bioactive compound as mentioned earlier in Fig. 2 (scaffolds 1–4), we decided to explore cyclization using various isothiocyanates. Hence, the amine conjugates **13** were condensed with selective isothiocyanates using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as an activating agent in anhydrous acetonitrile under microwave irradiation at 100 °C (5 bar) to furnish the benzoxazole conjugate **14** in 10 min. However, it has been observed that the same reaction required 10 h under conventional reflux conditions, which in turn reflects the advantages of microwave irradiation. After completion of the reaction, the reaction mixture was precipitated with cold ether and filtered through a fritted funnel to obtain benzoxazole conjugate **14** in good yields. The formation of the product involves the nucleophilic attack of the amine group on the isothiocyanate moiety to form intermediate '**a**'. For mechanistic investigation, intermediate '**a**' was isolated before addition of the coupling reagents and characterized (see the ESI†). The coupling agent (EDC) promotes further activation of the thiocarbonyl (C═S) moiety of intermediate '**a**', which after cyclization and electronic reorganization generate the target compounds **14** as depicted in Fig. 4. Additionally, the isolated intermediate '**a**' was also reacted separately with EDC, providing the same results, which supports the predicted mechanism.

The benzimidazolyl benzoxazoles were finally cleaved from the polymer support using a 1% solution of KCN in MeOH at room temperature within 12 h. The reaction mixture was concentrated and polymer support was precipitated by ether and removed by filtration. The filtrates were evaporated and subjected to HPLC analysis which indicated 76–98% crude purity of the title compounds. Finally, column chromatography purification afforded the benzimidazolyl benzoxazole derivatives **15** in good overall yields (Table 1). By utilizing the desired reaction sequence, we have synthesized various benzimidazolyl benzoxazole derivatives **15** with two diverse substitutions as shown in Table 1. The systematic

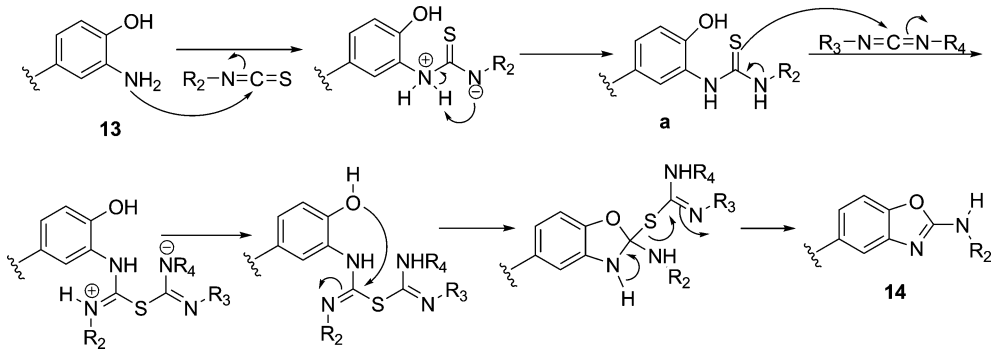


Fig. 4 Plausible mechanism towards the formation of benzimidazolyl benzoxazoles.

variation of amines and isothiocyanate components led to the structural diversity in the scaffold.

Monitoring the progress of a reaction on an insoluble polymer support throughout a multistep synthetic sequence is a relatively difficult task. The idea of having a polymer support possessing conventional solubility properties in organic solvents has been very well realized in the use of polyethylene glycols (PEG). A significant advantage of NMR protocols for soluble polymer supports is its nondestructive nature as compared to the customary "*cleave and characterize*" techniques used for solid-supported synthesis.[18] The comparative analysis of NMR spectra (aromatic region) of all the steps is depicted in Fig. 5. Formation of the anilide conjugates was confirmed by the appearance of NH signals at 9.18 ppm, whereas the additional signals emerged from the absorbance of Hd, He and Hf protons of 4-hydroxy-3-nitrobenzoic acid in spectrum B. Establishment of the benzimidazole ring was evident from the disappearance of the NH proton in spectrum C and Ha, Hb and Hc protons were shifted downfield due to the electron withdrawing nature of the benzimidazole moiety. Reduction of the nitro group was noticed from the substantial shifting of Hd and He protons to the upfield region at 7.00 and 6.81 ppm in spectrum D. Final cyclization with allylisothicyanate to **14j** generated the electron withdrawing benzoxazole derivatives, which was observed in spectrum E. Due to the electron withdrawing effect, the previously apparent upfield protons Hd and He at 7.0 and 6.81 ppm became deshielded and moved to 7.45 and 7.32 ppm. Finally, the cleavage of the product from the polymer support was confirmed by observing the absence of PEG signals around 4.4 ppm along with the slight downfield shift of all aromatic protons of **15j** in spectrum F.

Additionally, the structure of the final compounds was unambiguously confirmed by the X-ray crystallographic study. Fig. 6 depicts the ORTEP diagram of compound **15n**. The single crystal X-ray analysis of compound **15n** indicates that the two rings of the benzimidazole and benzoxazole moieties are anti-periplanar to each other and in angular orientation.

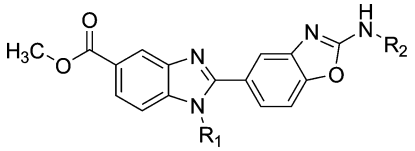
After the successful synthesis of the benzoxazole bis-heterocyclic library, our interest in the biological evaluation of these compounds deviated towards the VEGFR kinase inhibitors due to their structural resemblance with the potent KDR inhibitor[14] and potential VEGFR-3 inhibitor.[19] It is a well established fact in cancer biology that tumor growth depends on the expression of various growth factors associated with angiogenesis and lymphangiogenesis. Recent advanced studies on the identification of the roles of various VEGFRs showed that VEGFR-3 has been implicated as a key mediator of lymphangiogenesis in both normal biology and tumors.[20] Consequently, as a novel VEGFR target, we are intrigued to examine the new bis-heterocyclic scaffold for the inhibition of VEGFR-3.

Accordingly, the new benzimidazolyl linked benzoxazole derivatives **15a–p** were examined for *in vitro* inhibition of VEGFR-3 kinase in a cell-based assay in the concentration of 3 μM on H928 cell lines. Interestingly, after preliminary screening it has been found that all the compounds have shown moderate to high inhibition from 15% to 87% (Table 2). Gratifyingly, compounds **15c**, **15f**, **15h–k**, **15m** and **15n** show higher inhibitory activity against VEGFR-3. These selective compounds were further studied for the IC_{50} values and the respective data are specified in Table 2. The comparative biological studies point to compounds **15c**, **15m** and **15n** as the prominent compounds that inhibit receptor tyrosine kinase VEGFR-3. A generalization of this study suggests that the new benzimidazole linked benzoxazole bis-heterocyclic scaffold possesses potential to inhibit receptor tyrosine kinase VEGFR-3 and further extension of this study can definitely aid the development of novel cancer therapeutics.

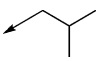
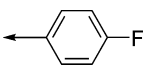
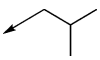
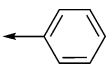
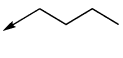
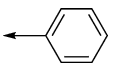
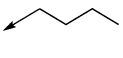
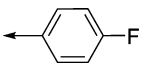
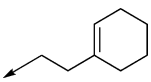
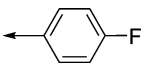
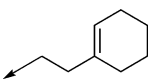
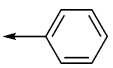
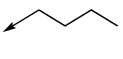
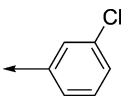
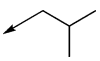
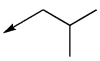
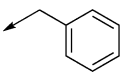
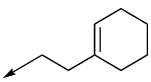
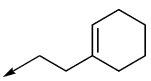
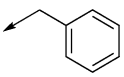
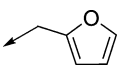
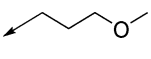
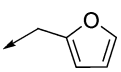
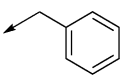
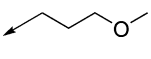
Conclusions

In conclusion, we have developed a new strategy for a rapid synthesis of novel substituted benzimidazole linked benzoxazole derivatives in a multistep process on soluble polymer support under focused microwave irradiation. The proposed bis-heterocyclic system was developed by constructing the benzoxazole ring over benzimidazole by sequential reactions, specifically condensation, reduction and cyclization with isothiocyanate. We have significantly applied the combination of soluble polymer support and microwave irradiation to increase the overall efficiency of the process through rapid reactions as well as purification by simple precipitation with ether. The efficacy of the novel benzimidazolyl benzoxazole derivatives was examined for VEGFR-3 tyrosine kinase inhibition activity. Preliminary screening results have shown that some of these compounds exhibited moderate to good inhibition against VEGFR-3, which is related to the invasion and migration of cancer cells. The bis-heterocyclic structural framework defined by the benzimidazole linked benzoxazole core and the ease of its access by microwave assisted soluble supported synthesis open up new avenues for the development of anti-angiogenic drugs.

Table 1 Synthesized benzo[*d*]oxazol-5-yl-1*H*-benzo[*d*]imidazole-5-carboxylates **15**



15

Comp.	R_1	R_2	Yield[a](%)	Purity[b](%)	LRMS[c]
15a			90	88	459
15b			85	89	441
15c			93	95	441
15d			91	97	459
15e			82	89	511
15f			92	96	493
15g			95	94	474
15h			82	80	405
15i			81	82	455
15j			89	94	457
15k			85	80	507
15l			95	98	457
15m			77	76	461
15n			79	77	467
15o			87	86	437
15p			96	98	417

[a] Yields were determined on the weight of purified samples (%). [b] HPLC analysis (UV detection at 254 nm) of the crude samples (%). [c] LRMS were detected with ESI ionization source.

Table 2 Percent inhibition of VEGFR-3 and IC_{50} (μM) value for selected compounds

Comp.	% inhibition of VEGFR-3[a] at 3 μM	IC_{50} (μM)[b]
15a	52	—
15b	40	—
15c	60	0.75
15d	29	—
15e	46	—
15f	69	1.19
15g	53	—
15h	76	1.03
15i	86	1.25
15j	63	1.18
15k	73	1.42
15l	15	—
15m	81	0.56
15n	87	0.70
15o	81	—
15p	22	—

[a] Results are expressed as the mean percent inhibition at 3 μM level. [b] For the selective compounds having inhibition more than 60%; values are an average of three individual determinations.

Experimental section

General methods

All reactions were performed under an inert atmosphere with unpurified reagents and dry solvents. Analytical thin-layer chromatography (TLC) was performed using 0.25 mm silica gel coated plates. Flash chromatography was performed using the indicated solvent and silica gel 60 (230–400 mesh). All the microwave experiments were performed in a Biotage initiator under optimized reaction conditions of power and pressure. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a 300 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) on the scale from an internal standard. High-resolution mass spectra (HRMS) were recorded on a JEOL TMS-HX 110 mass spectrometer. Normal phase HPLC was performed on a Shimadzu LC-10AT series machine with a Hypersil (250 × 4.6 mm) analytical column.

General procedure for the preparation of polymer-bound 3-(4-hydroxy-3-nitrobenzamido)-4-(substituted amino) carboxylates 11. To a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (139 mg, 0.67 mmol, 3.0 equiv) in *N*,*N*′-dimethylformamide (DMF) was added 4-hydroxy-3-nitrobenzoic acid **10** (122 g, 0.67 mmol, 3 equiv.) and 1-hydroxybenzotriazole (HOBt) (91 mg, 0.67 mmol, 3.0 equiv) in sequential order. The resulting slurry was stirred for 5 min at room temperature and then polymer (PEG 4000) anchored *o*-phenylene diamine **9** (1.0 g, 0.22 mmol, 1.0 equiv.) in DMF (5 mL) was added. The reaction mixtures were subsequently heated with stirring in a 10 mL microwave process vial for 25 min in the appropriate mode of pressure and temperature to obtain the polymer conjugate **11**. After completion of the reaction, the suspended byproducts were filtered through filter paper. The reaction mixtures were precipitated by slow addition of cold ether (50 mL) and the precipitated amide conjugates **11** were filtered through a fritted funnel. The crude product was washed in succession with ether (100 mL × 3) to remove the undesired impurities and dried for further steps.

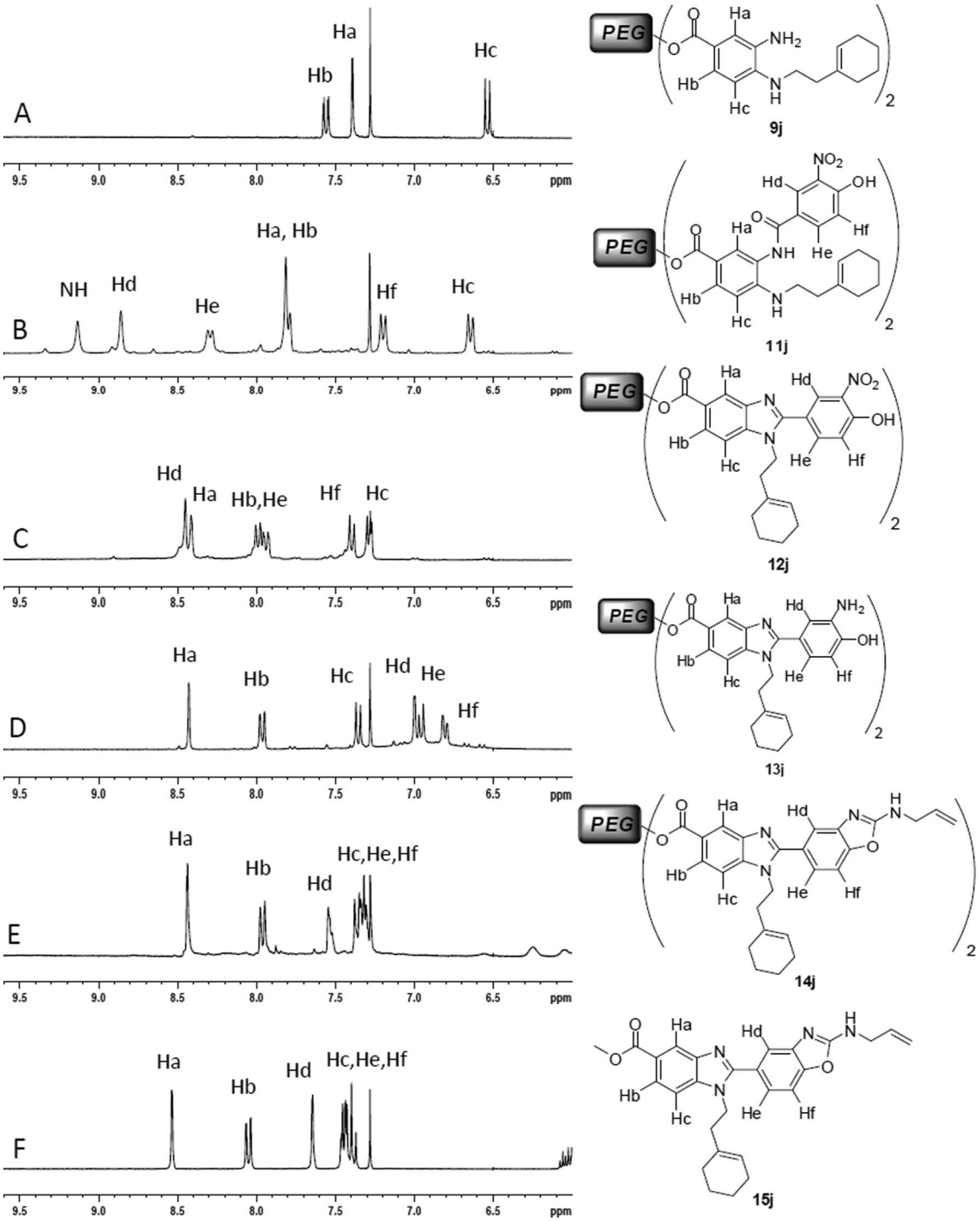


Fig. 5 Stepwise monitoring of multistep synthesis of benzimidazolyl benzoxazoles by proton NMR spectroscopy.

General procedure for the preparation of polymer-bound 2-(4-hydroxy-3-nitrophenyl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates 12. To a solution of polymer-bound 3-(4-hydroxy-3-nitrobenzamido)-4-(substituted amino) carboxylates **11** in 1,2-dichloroethane, trifluoroacetic acid (0.5 mL) and $MgSO_4$ (500 mg) were added and the mixture was subsequently heated with stirring in a 10 mL microwave process vial for 8 min in the appropriate mode of pressure and temperature. After completion of the reaction, the $MgSO_4$ was removed through Celite. The reaction mixtures were precipitated by slow addition of excess cold ether (100 mL) and filtered through a fritted funnel to obtain the polymer-bound 2-(4-hydroxy-3-nitrophenyl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates **12** in high purity.

General procedure for the preparation of polymer-bound 2-(3-amino-4-hydroxyphenyl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates 13. To a solution of **12** in methanol, Pd on charcoal (48 mg, 0.45 mmol, 1.0 equiv.) and ammonium formate (132 mg,

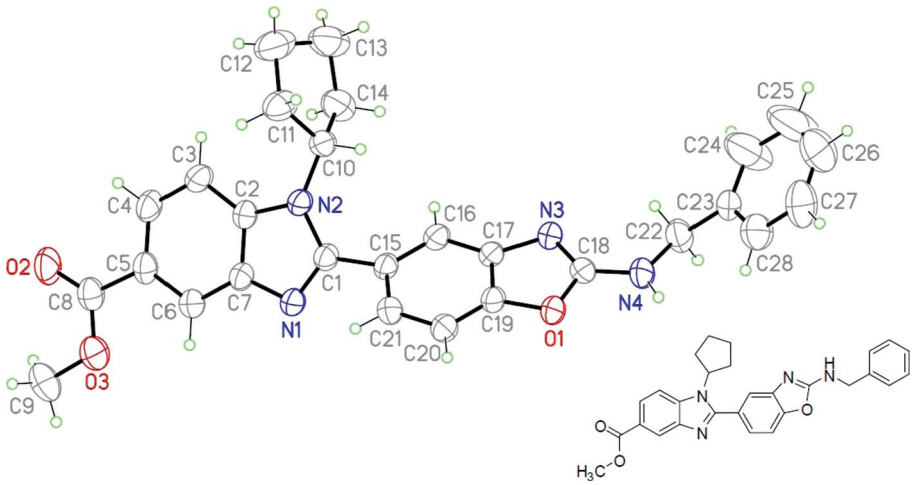


Fig. 6 ORTEP diagram of compound **15n**.

2.10 mmol, 10.0 equiv.) were added. The reaction mixture was subsequently heated with stirring in a 10 mL microwave process vial for 10 min in the appropriate mode of pressure and temperature until complete reduction of the nitro group which was evident from a color change (yellow to greenish blue) upon spotting on a TLC plate. After completion, the reaction mixtures were then subjected to centrifugation for removal of the Pd on charcoal and the supernatant liquid was concentrated by rotary evaporation to remove methanol. Dichloromethane (10 mL) was then added to salt out ammonium formate. The reaction mixtures were filtered through a fritted funnel to remove ammonium formate, to obtain the polymer-bound 2-(3-amino-4-hydroxyphenyl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates **13**.

General procedure for the preparation of polymer-bound 2-(2-(substituted amino) benzo[*d*]oxazol-5-yl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates 14. To a stirred solution of polymer-bound 2-(3-amino-4-hydroxyphenyl)-1-alkyl-1*H*-benzo[*d*]imidazole carboxylates conjugates **13** in CH_3CN (5 mL), various isothiocyanates (1.05 mmol, 5.0 equiv.) and *N*-3(dimethylaminopropyl)-3-ethyl carbodiimide (EDC) (163 mg, 1.05 mmol, 5.0 equiv) as an activating agent were added. The reaction mixtures were exposed under pressure to microwave irradiation for 10 min. Upon completion of cyclization by checking the NMR, the crude product mixtures were purified by precipitation with cold ether (100 mL × 3) and dried to obtain the conjugate **14** in high purity.

General procedure for the cleavage of polymer-bound substituted benzimidazolylbenzoxazoles 15. To a solution of polymer conjugates **14** in methanol (20 mL), KCN (100 mg) was added and stirred for 12 h at room temperature. After completion of the reaction, the crude product was precipitated with excess cold ether (100 mL), the polymer was filtered off and subjected to evaporation. The residue was dried under vacuum, and subjected to crude HPLC analysis with UV detection at $\lambda = 254$ nm (column: Sphereclone 5μ Si (250 × 4.6 mm); gradient: 35% ethyl acetate in hexane; flow rate: 1 mL min^{-1}). The slurry obtained was loaded onto a silica gel column and eluted with a mixture of ethyl acetate and hexane (1 : 4) to get the title compounds **15** in 77–96% overall yields.

2-(2-(4-Fluorophenylamino)benzo[*d*]oxazol-5-yl)-1-isobutyl-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15a. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (d, J = 1.5 Hz, 1H), 8.07 (dd, J = 8.5, 1.5 Hz, 1H), 7.98 (brs, 1H), 7.71 (d, J = 1.5 Hz, 1H), 7.64 (dd, J = 9.0 Hz, J_{HF} = 4.6 Hz, 1H), 7.47 (d, J = 8.5 Hz, 1H), 7.45 (dd, J = 8.2, 1.5 Hz, 1H), 7.36 (d, J = 8.2 Hz, 1H), 7.08 (dd, J_{HF} = 8.8 Hz, J = 7.4 Hz, 2H), 4.13 (d, J = 7.5 Hz, 2H), 3.98 (s, 3H), 2.11 (sept, J = 6.6 Hz, 1H), 0.74 (d, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.0, 159.8, 159.4 (d, $^1J_{CF}$ = 252.9 Hz), 156.5, 149.2, 143.2, 142.7, 139.3, 134.3, 126.7, 125.0, 124.7, 124.1, 122.4, 120.8 (d, $^3J_{CF}$ = 7.8 Hz), 118.1, 116.4 (d, $^2J_{CF}$ = 26.1 Hz), 110.7, 109.8, 52.5, 52.4, 29.4, 20.5, 20.4; IR (cm^{-1}, KBr): 3303, 1708; MS (ESI) m/z 459 (MH^+); HRMS (ESI, m/z) calcd for $C_{26}H_{23}FN_4O_3$: m/z 459.1832; Found 459.1834.

1-Isobutyl-2-(2-(phenylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo-[*d*]imidazole-5-carboxylic acid methyl ester 15b. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.57 (d, J = 1.5 Hz, 1H), 8.07 (dd, J = 8.5, 1.5 Hz, 1H), 7.97 (brs, 1H), 7.74 (d, J = 1.4 Hz, 1H), 7.67 (dd, J = 8.7, 1.2 Hz, 2H), 7.48 (dd, J = 8.3, 1.4 Hz, 1H), 7.47–7.39 (m, 4H), 7.14 (t, J = 7.4 Hz, 1H), 4.13 (d, J = 6.6 Hz, 2H), 3.98 (s, 3H), 2.13 (sext, J = 6.6 Hz, 1H), 0.73 (d, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 159.4, 156.5, 149.2, 145.4, 143.4, 142.9, 139.4, 138.0, 129.8, 126.9, 124.8, 124.3, 123.9, 122.6, 118.9, 118.3, 110.6, 109.9, 53.2, 52.5, 29.2, 20.5, 20.4; IR (cm^{-1}, KBr): 3434, 1712; MS (ESI) m/z 441 (MH^+); HRMS (ESI, m/z) calcd for $C_{26}H_{24}N_4O_3$: m/z 441.1927; Found 441.1925.

1-Butyl-2-(2-(phenylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo-[*d*]imidazole-5-carboxylic acid methyl ester 15c. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.48 (s, 1H), 8.58 (s, 1H), 8.08 (d, J = 8.4 Hz, 1H), 7.71 (s, 1H), 7.66 (d, J = 7.8 Hz, 2H), 7.46 (d, J = 8.6 Hz, 1H), 7.42 (d, J = 8.6 Hz, 1H), 7.37–7.29 (m, 3H), 7.07 (t, J = 7.2 Hz, 1H), 4.27 (t, J = 7.1 Hz, 2H), 3.97 (s, 3H), 1.74 (quint, J = 7.1 Hz, 2H), 1.27–1.22 (m, 2H), 0.83 (t, J = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 159.9, 156.3, 149.3, 143.4, 142.8, 139.1, 138.4, 129.7, 126.2, 124.7, 123.9, 123.7, 123.2, 122.4, 119.1, 117.8, 110.4, 109.8, 52.6, 45.1, 32.1, 20.3, 13.9; IR (cm^{-1}, KBr): 3436, 1714; MS (ESI) m/z 441 (MH^+); HRMS (ESI, m/z) calcd for $C_{26}H_{24}N_4O_3$: m/z 441.1927; Found 441.1924.

1-Butyl-2-(2-(4-fluorophenylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15d. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.25 (s, 1H), 8.57 (s, 1H), 8.08 (d, J = 8.5 Hz, 1H), 7.68 (s, 1H), 7.63 (dd, J = 8.5 Hz, J_{HF} = 4.6 Hz, 2H), 7.47 (d, J = 8.5 Hz, 1H), 7.39 (d, J = 8.5 Hz, 1H), 7.26 (d, J = 8.5 Hz, 1H), 7.05 (dd, J = 8.5 Hz, J_{HF} = 8.4 Hz, 2H), 4.27 (t, J = 7.2 Hz, 2H), 3.98 (s, 3H), 1.78 (quint, J = 7.2 Hz, 1H), 1.26–1.19 (m, 2H), 0.84 (t, J = 7.2 Hz, 3H); ^{19}F NMR (282.4 MHz, $CDCl_3$) δ –119.0; ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 160.9, 159.9 (d, $^1J_{CF}$ = 260.6 Hz), 156.2, 149.3, 143.3, 142.7, 139.1, 134.5, 126.1, 125.1, 125.0, 124.6, 122.3, 120.9 (d, $^3J_{CF}$ = 4.0 Hz), 118.1, 116.5 (d, $^2J_{CF}$ = 23.1 Hz), 111.5, 110.4, 52.6, 45.1, 32.1, 20.3, 13.9; IR (cm^{-1}, KBr): 3303, 1709; MS (ESI) m/z 459 (MH^+); HRMS (ESI, m/z) calcd for $C_{26}H_{23}FN_4O_3$: m/z 459.1832; Found 459.1831.

1-(2-Cyclohexenylethyl)-2-(2-(4-fluorophenylamino)benzo[*d*]-oxazol-5-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15e. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (brs, 2H), 8.08 (d, J = 8.5 Hz, 1H), 7.72 (s, 1H), 7.62 (dd, J = 8.6 Hz, J_{HF} = 4.5 Hz, 2H), 7.49 (d, J = 8.2 Hz, 2H), 7.33 (d, J = 8.2 Hz, 1H), 7.09 (dd, J = 8.6 Hz, J_{HF} = 8.5 Hz, 2H), 5.19 (m, 1H), 4.39 (t, J = 7.2 Hz, 2H), 3.99 (s, 3H), 2.35 (t, J = 7.2 Hz, 2H), 1.83–1.75 (m, 4H), 1.44–1.34 (m, 4H); ^{19}F NMR (282.4 MHz, $CDCl_3$) δ –118.9; ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 161.1, 159.7, 159.4 (d, $^1J_{CF}$ = 262.2 Hz), 156.2, 149.3, 143.3, 142.8, 139.2, 134.2, 133.4, 126.5, 125.1, 124.9, 124.7, 124.2, 122.5, 120.9 (d, $^3J_{CF}$ = 7.5 Hz), 118.0, 116.4 (d, $^2J_{CF}$ = 30.0 Hz), 110.4, 109.8, 52.6, 44.2, 38.1, 28.6, 25.5, 22.9, 22.3; IR (cm^{-1}, KBr): 3446, 1716; MS (ESI) m/z 511 (MH^+); HRMS (ESI, m/z) calcd for $C_{30}H_{27}FN_4O_3$: m/z 511.2145; Found 511.2142.

1-(2-Cyclohexenylethyl)-2-(2-(phenylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15f. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.00 (brs, 1H), 8.57 (d, J = 1.5 Hz, 1H), 8.08 (dd, J = 8.5, 1.5 Hz, 1H), 7.73 (s, 1H), 7.66 (d, J = 8.2 Hz, 2H), 7.45 (d, J = 8.5 Hz, 2H), 7.44–7.30 (m, 3H), 7.10 (t, J = 8.2 Hz, 1H), 5.18 (m, 1H), 4.37 (t, J = 7.2 Hz, 2H), 3.98 (s, 3H), 2.34 (t, J = 7.2 Hz, 2H), 1.81–1.73 (m, 4H), 1.44–1.41 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 159.8, 156.3, 149.2, 143.3, 142.8, 139.2, 138.3, 133.4, 129.7, 126.4, 125.1, 124.9, 124.7, 123.8, 123.6, 122.4, 119.0, 117.9, 110.5, 109.8, 52.6, 43.1, 38.1, 28.6, 25.5, 22.9, 21.8; IR (cm^{-1}, KBr): 3421, 1712; MS (ESI) m/z 493 (MH^+); HRMS (ESI, m/z) calcd for $C_{30}H_{28}N_4O_3$: m/z 493.2240; Found 493.2242.

1-Butyl-2-(2-(3-chlorophenylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15g. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.20 (brs, NH), 8.56 (s, 1H), 8.09 (d, J = 8.5 Hz, 1H), 7.80 (s, 1H), 7.72 (s, 1H), 7.55 (d, J = 8.2 Hz, 1H), 7.48 (d, J = 8.5 Hz, 1H), 7.41 (d, J = 8.2, 1H), 7.30–7.25 (m, 2H), 7.05 (d, J = 7.8 Hz, 1H), 4.29 (t, J = 7.4 Hz, 2H), 3.99 (s, 3H), 1.80 (quint, J = 7.4 Hz, 2H), 1.30–1.22 (m, 2H), 0.85 (t, J = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.0, 159.1, 156.2, 149.2, 143.2, 142.7, 139.6, 139.1, 135.3, 130.7, 126.2, 125.1, 124.8, 124.3, 123.6, 122.3, 118.8, 118.2, 116.8, 110.4, 109.9, 52.6, 45.1, 32.1, 20.3, 13.9; IR (cm^{-1}, KBr): 3399, 1747; MS (EI) m/z 474 (M^+); HRMS (ESI, m/z) calcd for $C_{26}H_{23}ClN_4O_3$: m/z 475.1537; Found 475.1533.

2-(2-(Allylamino)benzo[*d*]oxazol-5-yl)-1-isobutyl-1*H*-benzo[*d*]-imidazole-5-carboxylic acid methyl ester 15h. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, J = 1.4 Hz, 1H), 8.04 (dd, J = 8.5, 1.4 Hz, 1H), 7.60 (s, 1H), 7.42 (d, J = 8.5 Hz, 1H), 7.38–7.34 (m, 2H), 6.11 (brs, 1H), 5.99 (m, 1H), 5.33 (dd, J = 17.0, 1.1 Hz, 1H), 5.22 (dd, J = 10.1, 1.1 Hz, 1H), 4.12 (m, 4H), 3.96 (s, 3H), 2.10 (sept, J = 6.6 Hz, 1H), 0.71 (d, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 163.3, 156.6, 149.9, 143.8, 142.9, 139.4, 133.9, 126.6, 124.8, 124.5, 123.3, 122.6, 117.6, 117.4, 110.6, 109.6, 52.5, 52.4, 45.8, 29.1, 20.3; IR (cm^{-1}, KBr): 3278, 1710; MS (ESI) m/z 405 (MH^+); HRMS (ESI, m/z) calcd for $C_{23}H_{24}N_4O_3$: m/z 405.1927; Found 405.1929.

2-(2-(Benzylamino)benzo[*d*]oxazol-5-yl)-1-isobutyl-1*H*-benzo-[*d*]imidazole-5-carboxylic acid methyl ester 15i. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, J = 1.6 Hz, 1H), 8.04 (dd, J = 8.5, 1.6 Hz, 1H), 7.56 (d, J = 1.1 Hz, 1H), 7.44 (d, J = 8.5 Hz, 1H), 7.42–7.30 (m, 7H), 5.97 (t, J = 6.0 Hz, 1H), 4.72 (d, J = 6.0 Hz, 2H), 4.13 (d, J = 7.5 Hz, 2H), 3.97 (s, 3H), 2.10 (sept, J = 6.6 Hz, 1H), 0.73 (d, J = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 163.2, 156.5, 149.9, 143.8, 142.9, 139.4, 137.8, 129.3, 128.4, 128.1, 126.8, 124.8, 124.5, 123.5, 122.6, 117.6, 110.6, 109.0, 52.5, 52.4, 47.6, 29.2, 20.3; IR (cm^{-1}, KBr): 3210, 1705; MS (ESI) m/z 455 (MH^+); HRMS (ESI, m/z) calcd for $C_{27}H_{26}N_4O_3$: m/z 455.2083; Found 455.2085.

2-(2-(Allylamino)benzo[*d*]oxazol-5-yl)-1-(2-Cyclohexenylethyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15j. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (d, J = 1.5 Hz, 1H), 8.05 (dd, J = 8.5, 1.5 Hz, 1H), 7.64 (d, J = 1.5 Hz, 1H), 7.46 (dd, J = 8.2, 1.5 Hz, 1H), 7.43 (d, J = 8.5 Hz, 1H), 7.38 (d, J = 8.2 Hz, 1H), 6.00 (m, 1H), 5.86 (m, 1H), 5.35 (dd, J = 17.1, 1.3 Hz, 1H), 5.24 (dd, J = 10.3, 1.3 Hz, 1H), 5.18 (m, 1H), 4.36 (t, J = 7.2 Hz, 2H), 4.17–4.14 (m, 2H), 3.97 (s, 3H), 2.33 (t, J = 7.2 Hz, 2H), 1.81–1.74 (m, 4H), 1.45–1.43 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 163.2, 156.3, 150.1, 143.9, 142.9, 139.3, 133.9, 133.4, 126.4, 124.9, 124.8, 124.5, 123.3, 122.6, 117.4, 117.3, 110.3, 109.6, 52.5, 45.8, 44.1, 38.1, 28.6, 25.5, 22.9, 22.3; IR (cm^{-1}, KBr): 3424, 1714; MS (ESI) m/z 457 (MH^+); HRMS (ESI, m/z) calcd for $C_{27}H_{28}N_4O_3$: m/z 457.2240; Found 457.2239.

2-(2-(Benzylamino)benzo[*d*]oxazol-5-yl)-1-(2-Cyclohexenylethyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15k. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, J = 1.4 Hz, 1H), 8.05 (dd, J = 8.5, 1.4 Hz, 1H), 7.54 (d, J = 1.3 Hz, 1H), 7.43 (d, J = 8.5 Hz, 1H), 7.40–7.23 (m, 7H), 6.65 (m, 1H), 5.18 (m, 1H), 4.68 (d, J = 4.9 Hz, 2H), 4.34 (t, J = 7.2 Hz, 2H), 3.96 (s, 3H), 2.32 (t, J = 7.2 Hz, 2H), 1.81–1.73 (m, 4H), 1.45–1.42 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.2, 163.4, 156.3, 150.1, 143.9, 142.9, 139.3, 137.9, 133.4, 129.2, 128.2, 128.0, 126.3, 125.0, 124.8, 124.5, 123.2, 122.5, 117.2, 110.3, 109.6, 52.5, 47.5, 44.1, 38.1, 28.6, 25.5, 23.0, 22.3; IR (cm^{-1}, KBr): 3209, 1714; MS (ESI) m/z 507 (MH^+); HRMS (ESI, m/z) calcd for $C_{31}H_{30}N_4O_3$: m/z 507.2396; Found 507.2395.

1-Cyclopentyl-2-(2-(furan-2-ylmethylamino)benzo[*d*]oxazol-5-yl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15l. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.00 (d, J = 8.6 Hz, 1H), 7.61 (s, 1H), 7.54 (d, J = 8.6 Hz, 1H), 7.45–7.39 (m, 3H), 6.42–6.32 (m, 2H), 5.62 (t, J = 5.5 Hz, 1H), 4.99 (quint, J = 8.9 Hz, 1H), 4.71 (d, J = 5.5 Hz, 2H), 3.98 (s, 3H), 2.36–2.29 (m, 2H), 2.14–2.06 (m, 4H), 1.75–1.73 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.0, 163.0, 156.7, 150.9, 150.1, 143.6, 143.0, 142.9, 136.9, 126.6, 124.6, 124.0, 123.6, 122.9, 117.5, 111.9, 110.9, 109.7, 108.4, 58.1, 52.5, 40.4, 30.8, 25.6; IR (cm^{-1}, KBr): 3423, 1714; MS (ESI) m/z 457

(MH$^+$); HRMS (ESI, *m/z*) calcd for $C_{26}H_{24}N_4O_4$: *m/z* 457.1876; Found 457.1878.

2-(2-(Furan-2-ylmethylamino)benzo[*d*]oxazol-5-yl)-1-(3-methoxypropyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15m. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, J = 1.5 Hz, 1H), 8.05 (dd, J = 8.5, 1.5 Hz, 1H), 7.67 (d, J = 1.4 Hz, 1H), 7.50 (d, J = 8.5 Hz, 1H), 7.46–7.36 (m, 3H), 6.35 (dd, J = 5.6, 4.9 Hz, 2H), 6.13 (brs, 1H), 4.70 (s, 2H), 4.42 (t, J = 7.1 Hz, 2H), 3.97 (s, 3H), 3.24 (t, J = 6.0 Hz, 2H), 3.21 (s, 3H), 1.99 (quint, J = 6.2 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 162.9, 156.1, 150.9, 150.1, 143.7, 143.0, 142.8, 139.4, 125.3, 124.7, 124.6, 123.4, 122.5, 117.4, 110.9, 110.3, 109.7, 108.5, 69.1, 59.0, 52.5, 42.2, 40.4, 30.4; IR (cm^{-1}, KBr): 3211, 1708; MS (ESI) *m/z* 461 (MH$^+$); HRMS (ESI, *m/z*) calcd for $C_{25}H_{24}N_4O_5$: *m/z* 461.1825; Found 461.1822.

2-(2-(Benzylamino)benzo[*d*]oxazol-5-yl)-1-cyclopentyl-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15n. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.51 (d, J = 1.5 Hz, 1H), 8.00 (dd, J = 8.6, 1.5 Hz, 1H), 7.54 (d, J = 1.0 Hz, 1H), 7.53 (d, J = 8.6 Hz, 1H), 7.42–7.34 (m, 7H), 6.16 (t, J = 5.4 Hz, 1H), 4.98 (quint, J = 8.5 Hz, 1H), 4.71 (d, J = 5.4 Hz, 2H), 3.97 (s, 3H), 2.35–2.26 (m, 2H), 2.13–2.03 (m, 4H), 1.77–1.73 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 163.3, 156.7, 150.1, 143.7, 143.6, 137.8, 136.9, 129.3, 128.3, 128.0, 126.6, 124.5, 124.0, 123.5, 122.9, 117.4, 111.9, 109.6, 58.1, 52.5, 47.5, 30.8, 25.6; IR (cm^{-1}, KBr): 3266, 1712; MS (ESI) *m/z* 467 (MH$^+$); HRMS (ESI, *m/z*) calcd for $C_{28}H_{26}N_4O_3$: *m/z* 467.2083; Found 467.2080. Elemental analysis calcd (%) for $C_{28}H_{26}N_4O_3$: C 72.09, H 5.62, N 12.01; found: C 72.05, H 5.68, N 11.81, (O calcd 10.29, found 10.41).

2-(2-(Isobutylamino)benzo[*d*]oxazol-5-yl)-1-(3-methoxypropyl)-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15o. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, J = 1.5 Hz, 1H), 8.05 (dd, J = 8.5, 1.5 Hz, 1H), 7.64 (d, J = 1.5 Hz, 1H), 7.49 (d, J = 8.5 Hz, 1H), 7.47 (dd, J = 8.2, 1.5 Hz, 1H), 7.38 (d, J = 8.2 Hz, 1H), 5.50 (m, 1H), 4.42 (t, J = 6.9 Hz, 2H), 3.95 (s, 3H), 3.34 (m, 2H), 3.27 (t, J = 6.2 Hz, 2H), 3.21 (s, 3H), 2.18 (m, 1H), 2.02–1.97 (m, 2H), 1.03 (d, J = 6.7 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.2, 163.5, 156.2, 150.0, 145.4, 144.0, 142.9, 139.5, 124.9, 124.6, 123.1, 122.5, 117.1, 110.2, 109.5, 69.2, 59.0, 52.5, 51.0, 42.2, 30.3, 28.9, 20.4; IR (cm^{-1}, KBr): 3426, 1712; MS (ESI) *m/z* 437 (MH$^+$); HRMS (ESI, *m/z*) calcd for $C_{24}H_{28}N_4O_4$: *m/z* 437.2189; Found 437.2190.

2-(2-(Allylamino)benzo[*d*]oxazol-5-yl)-1-Cyclopentyl-1*H*-benzo[*d*]imidazole-5-carboxylic acid methyl ester 15p. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (d, J = 1.5 Hz, 1H), 8.00 (dd, J = 8.6, 1.5 Hz, 1H), 7.58 (d, J = 1.0 Hz, 1H), 7.53 (d, J = 8.6 Hz, 1H), 7.42–7.37 (m, 2H), 6.01 (m, 1H), 5.64 (m, 1H), 5.36 (dd, J = 17.1, 1.2 Hz, 1H), 5.26 (dd, J = 10.3, 1.2 Hz, 1H), 4.98 (quint, J = 8.5 Hz, 1H), 4.16 (t, J = 5.5 Hz, 2H), 3.97 (s, 3H), 2.39–2.26 (m, 2H), 2.14–2.06 (m, 4H), 1.77–1.73 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 163.1, 156.7, 150.0, 143.8, 143.7, 136.9, 133.9, 126.7, 125.0, 124.0, 123.6, 122.9, 117.5, 117.4, 111.9, 109.7, 58.1, 52.5, 45.8, 30.8, 25.6; IR (cm^{-1}, KBr): 3413, 1714; MS (ESI) *m/z* 417 (MH$^+$); HRMS (ESI, *m/z*) calcd for $C_{24}H_{24}N_4O_3$: *m/z* 417.1927; Found 417.1926.

KIRA ELISA assay (*in vitro* method for detecting VEGFR-3 activity). H928 cells (2×10^5) in 100 µl medium were added to each well in a flat bottom 24-well culture plate and cultured overnight at 37 °C in 5% CO_2. After the supernatants were removed, the cells were serum-starved for 24 h. A medium containing a test compound was added into each well and the cell culture was incubated for 30 min before it was stimulated by recombinant VEGF-C for 15 min. After the supernatants were removed, 100 µl of a lysis buffer were added into each well to lyse the cells and solubilize the VEGFR-3. The lysis buffer included 150 mM NaCl containing 50 mM Hepes (Genentech media prep), 0.5% Triton-X100 (Genentech media prep), 0.01% thimerosol, 30 kIU/ml aprotinin (ICN Biochemicals, Aurora, Ohio), 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), and 2 mM sodium orthovanadate. The plate was then put on a plate shaker (Bellco Instruments Vineland, N.J.) and the substance in each well of the plate underwent mixing for 60 minutes at room temperature. While the cells get soluble, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4 °C with the affinity-purified polyclonal anti-VEGFR-3 (2.5 mg/well block buffer (PBS containing 0.5% BSA and 0.01% thimerosol)) for 60 min at room temperature with gentle agitation. The anti-VEGFR-3 coated plate was subsequently washed twice with a wash buffer (PBS containing 0.05% Tween 20 and 0.01% thimerosol). The lysate containing solubilized VEGFR-3 from the cell culture microtiter well was transferred (85 µl/well) to the anti-VEGFR-3 coated ELISA plate and incubated for 2 h at room temperature with gentle agitation. The unbound receptors were removed by washing with a wash buffer. 100 µl of biotinylated 4G10 (antiphosphotyrosine) diluted to 0.2 µg ml^{-1} in dilution buffer (PBS containing 0.5% BSA, 0.05% Tween 20, 5 mM EDTA, and 0.01% thimerosol) were added into each well. After incubation for 2 h at room temperature, the plates were washed and 100 µl HRP-conjugated streptavidin (Zymed Laboratories, San Francisco, Calif.) diluted 1 : 2000 in dilution buffer was further added. After the free avidin conjugate was washed away, 100 µl freshly prepared substrate solution (tetramethyl benzidine, TMB) was added to each well. The reaction was allowed to proceed for 10 min and the color development was stopped by the addition of 100 µl/well 1.0 M H_3PO_4. The absorbance at 450 nm and the absorbance at a reference wavelength of 650 nm ($A_{450/650}$) were measured using an ELISA reader and the data were repeated 3 times. The inhibition efficacy of each test compound is expressed as an inhibition percentage calculated according to following formula: $1 - [(C - A)/(B - A)] \times 100$. In this formula, A is the basal amount of phosphotyrosine detected in a blank control, B is the amount of phosphotyrosine detected with VEGFR-C only, and C is the amount of phosphotyrosine detected with a test compound and VEGF-C. For IC_{50} generation, the final compound concentrations ranged from 0.1 µM to 10 µM.

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