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Synthesis and evaluation of naphthoflavones as a new class of non purine xanthine oxidase inhibitors

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This Letter is dedicated to Dr. K. L. Dhar on the occasion of his 78th birthday.

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ABSTRACT

In view of reported xanthine oxidase inhibitory potential of naphthopyrans and flavones, naphthoflavones as hybrids of the two were designed, synthesized and evaluated for in vitro xanthine oxidase inhibitory activity in the present study. The results of the assay revealed that the naphthoflavones possess promising inhibitory potential against the enzyme with IC_{50} values ranging from 0.62 to 41.2 μM. Structure activity relationship indicated that the nature and placement of substituents on the phenyl ring at 2nd position remarkably influences the inhibitory activity. Substitution of halo and nitro groups at *ortho* and *para* position of the phenyl ring (2nd position) remarkably favored the activity. **NF-4** with *p*-fluoro phenyl ring was the most potent inhibitor with IC_{50} value of 0.62 μM. Enzyme kinetics study was also performed to investigate the inhibition mechanism and it was found that the naphthoflavones displayed mixed type inhibition. The basis of significant inhibition of xanthine oxidase by **NF-4** was rationalized by molecular modeling studies.

Oxidative hydroxylation of hypoxanthine and xanthine catalyzed by xanthine oxidase to produce uric acid and reactive oxygen species leads to many diseases like gout and at least symptoms of diseases like oxidative damage to the tissue.[1–3] Therefore the selective inhibition of XO may result in broad spectrum chemotherapeutic for gout, cancer, inflammation and oxidative damage.[2–4] Allopurinol,[3,4] 2-alkyl hypoxanthines,[5] pterin and 6-formylpterin[6] represents the class of purine based xanthine oxidase inhibitors. All these inhibitors have been successfully utilized and have proved their inhibitory potential towards the enzyme. However these purine based inhibitors have been reported to be associated with Steven Johnson syndrome and worsening of renal function induced in some of the patients.[2–4] Keeping in view these side effects, we have been actively involved in the design of some non purine xanthine oxidase inhibitors in the recent past such as *n*-acetyl pyrazolines,[7] β-acetamido compounds,[8] azaflavones[9], naphthopyrans[10] and 4,6-diaryl/heteroarylpyrimidin-2(1*H*)-ones.[11]

Flavones represents the class of secondary metabolites possessing diverse array of biological activities.[7,12–16] Among the reported biological attributes, there are numerous reports on the xanthine oxidase inhibitory potential of flavones.[9,14–16] The structure activity relationship of flavones for xanthine oxidase inhibition has been extensively explored. SAR study revealed that a substituent free C-3 and overall planarity are two of the essential structural features required for xanthine oxidase inhibition of flavones.[14–16] Keeping these structural features intact, azaflavones were earlier designed via bioisosteric replacement of benzopyrans with quinolones by our research group.[9] The most potent azaflavone was found to possess significant inhibitory potential against the enzyme with an IC_{50} value of 6.24 μM.

Recently we synthesised and evaluated a series of naphthopyrans for in vitro xanthine oxidase inhibition in view of some of the potent non purine xanthine oxidase inhibitors possessing benzopyran skeleton. The results of the study proved that naphthyl moiety is an effective surrogate for the hydroxy substituted fused benzene ring in benzopyrans. The potent inhibitory potential of some naphthopyrans was attributed to the interactions of naphthyl ring as indicated by molecular modeling studies.[10]

With this background, naphthoflavones as hybrids of the naphthopyrans and flavones were designed keeping intact the two most important structural requisites for flavones, that is, a substituent free C-3 and overall planarity. Figure 1 represents the strategy for the design of naphthoflavones as xanthine oxidase inhibitors.

The hybrids were synthesized via Scheme 1. α-Naphthol was subjected to fries rearrangement as per the method reported by

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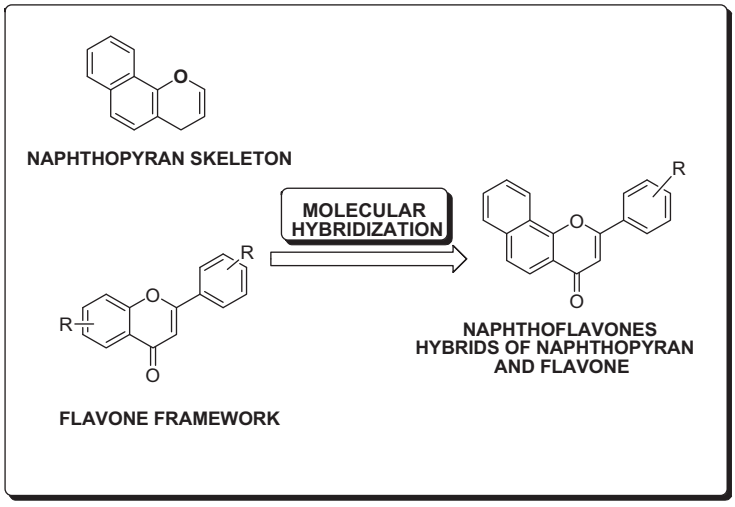
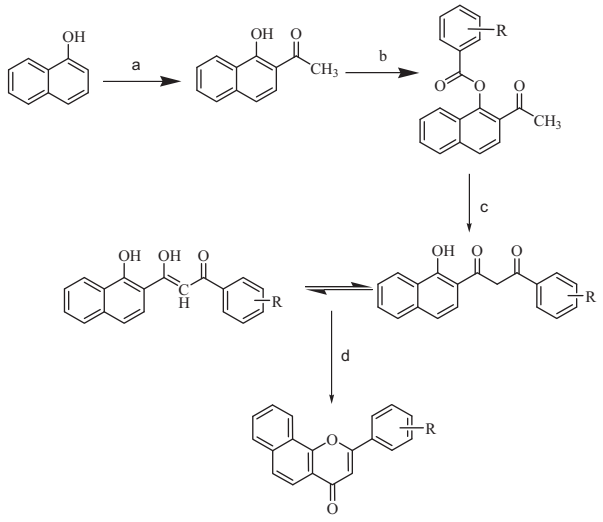


Figure 1. Design Strategy.



Scheme 1. Reagents and conditions: (a) MW, $ZnCl_2$, CH_3COOH, 20 min; (b) benzoyl chloride, pyridine, stirring rt, 1 h; (c) KOH, pyridine, warm, 30 min; (d) a drop of conc. H_2SO_4, CH_3COOH, reflux, 1 h.

Table 1
Structures of naphthoflavones along with IC_{50} values

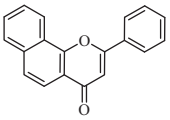
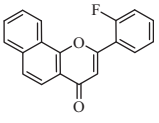
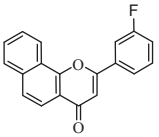
Code	Structure	IC_{50} (μM)
NF-1		32.5
NF-2		4.94
NF-3		23.6

Table 1 (*continued*)

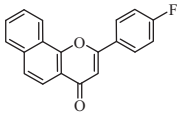
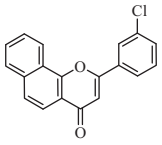
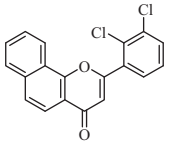
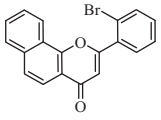
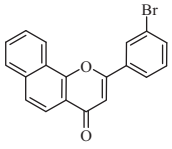
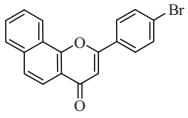
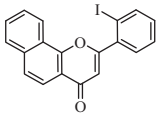
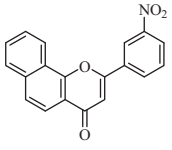
Code	Structure	IC_{50} (μM)
NF-4		0.62
NF-5		27.5
NF-6		41.2
NF-7		16.8
NF-8		31.2
NF-9		8.93
NF-10		19.4
NF-11		25.6

Table 1 (*continued*)

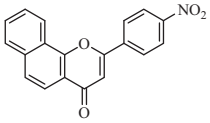
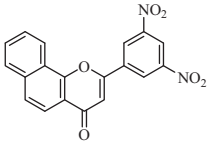
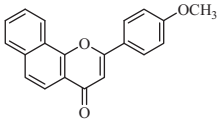
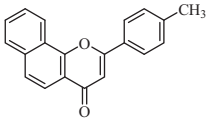
Code	Structure	IC_{50} (μM)
NF-12		1.95
NF-13		37.9
NF-14		18.9
NF-15		21.3
Apigenin		1.11
Allopurinol		8.69

Table 2
Inhibition of xanthine oxidase by potent compounds at 5 different concentrations

Code	1 μM	5 μM	10 μM	25 μM	50 μM	IC_{50} (μM)
NF-2	43.87	49.21	57.27	71.87	92.61	4.94
NF-4	49.39	52.10	61.67	78.51	99.47	0.62
NF-9	41.98	41.56	52.21	72.30	90.23	8.93
NF-12	47.76	50.13	61.18	73.83	94.27	1.95

Naeimi et al.[17] to yield 2-acetyl naphthol (1) (characterized by the appearance of a singlet for D_2O exchangeable proton at 14.01 ppm) which was further benzoylated using various substituted benzoyl-chlorides. The benzoylated product was then subjected to Baker Venkataraman rearrangement.[18] The Baker Venkataraman rearranged product existed in enol form (confirmed by the appearance of singlets for two D_2O exchangeable protons at 15.2 and 13.68 ppm along with the vinylic proton α to carbonyl which appeared as a merged signal in a multiplet at 7.26–7.36 ppm). The rearranged product was then cyclized by treatment with sulfuric acid to yield the desired naphthoflavones. All the reactions proceeded smoothly with diverse benzoylchlorides and products were obtained in good yields. No Retro-Diels fragmentation was observed for naphthoflavones in the mass spectrum.

The structures of the synthesized compounds were elucidated by ^1H NMR, ^{13}C NMR. All spectral data were in accordance with assumed structures.[18]

In vitro screening of the naphthoflavones using bovine milk xanthine oxidase (grade 1, ammonium sulfate suspension) enzymatic assay was performed as described in the literature.[19] Apigenin (one of the most potent flavone as xanthine oxidase inhibitor)[2] and allopurinol[20] were employed as reference inhibitor. The results of the in vitro assay (Table 1) indicated that naphthoflavones possessed significant xanthine oxidase inhibitory activity with IC_{50} value ranging from 0.62 to 41.2 μM. Naphthoflavones **NF-2**, **NF-4**, **NF-12** were found to be endowed with significant activity with IC_{50} <5 μM. **NF-4** was the most potent of the series displaying an IC_{50} value of 0.62 μM followed by **NF-2** and **NF-12** with an IC_{50} value of 4.94 and 1.95 μM. The in vitro screening of the naphthoflavones revealed some interesting aspects about the structure inhibitory relationship. The inhibitory potential of the compounds was sensitive towards the nature as well as positioning of the substituents on the phenyl ring at 2nd position. Compounds possessing substituted phenyl rings (any substitution whether electron withdrawing and electron donating) were more active than the compounds with an unsubstituted phenyl ring (Compare **NF-1** with **NF-2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, **14**, **15**). However only the naphthoflavones with disubstituted phenyl ring were less potent than the inhibitors with unsubstituted phenyl ring (compare **NF-1** with **NF-6** and **13**). Overall the preference order of substituents (monosubstitution) is as follows: halo and nitro > methoxy > methyl > hydrogen. Inhibitors with fluoro and nitro (*ortho* and *para*) substituted phenyl rings were the most active (**NF-2**, **4**, **12**) as they displayed IC_{50} value of <5 μM. Weak inhibitory potential was displayed by compounds with *meta* substituted phenyl rings as compared to compounds with *ortho* and *para* substituted phenyl rings. The decline in activity by the placement of *meta* substituted phenyl rings was evident from the IC_{50} values of **NF-3** (with *m*-fluoro phenyl, IC_{50} = 23.6 μM), **NF-4** (with *p*-fluoro phenyl, IC_{50} = 0.62 μM) and **NF-2** (with *o*-fluoro phenyl, IC_{50} = 4.94) as well as from the the IC_{50} values of **NF-8** (with *m*-bromo phenyl, IC_{50} = 31.2 μM), **NF-7** (with *o*-bromo phenyl, IC_{50} = 16.8 μM) and

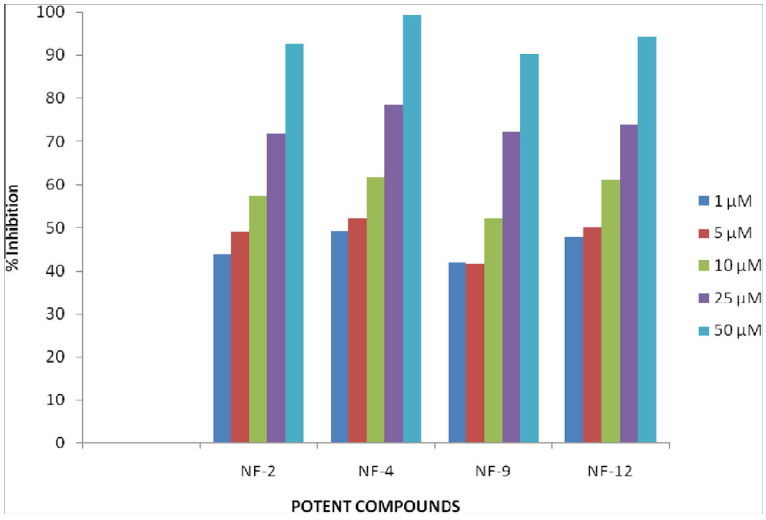


Figure 2. % Age inhibition of potent naphthoflavones.

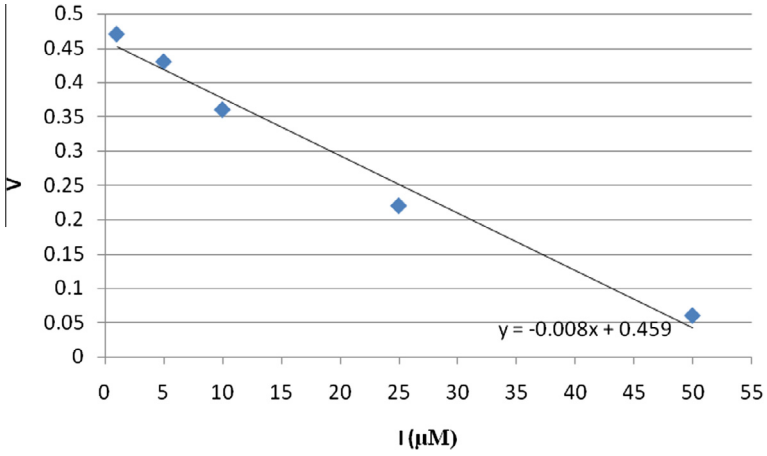


Figure 3. Plot of *V* versus [*I*] for **NF-4**.

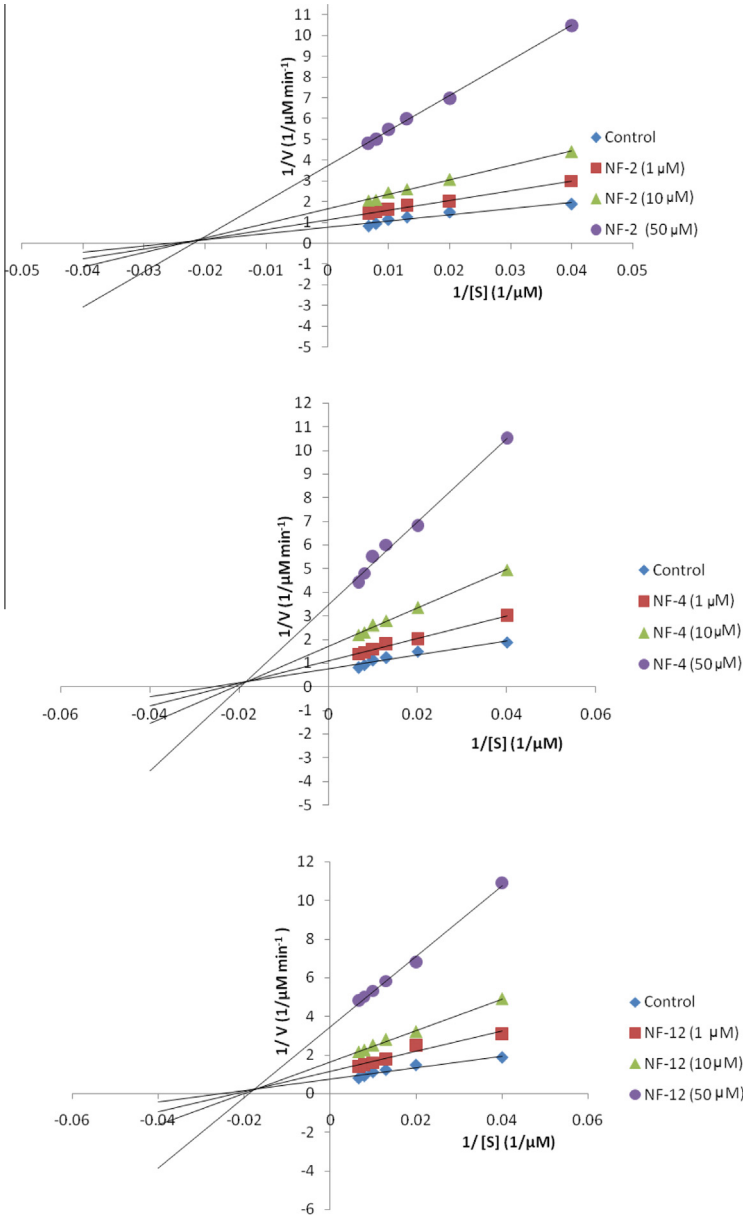


Figure 4. Lineweaver Burk plots.

NF-9 (with *p*-bromo phenyl, IC_{50} = 8.93 μM). **NF-4** was almost 36 folds and **NF-2** was 5 folds more active than **NF-3**. Similar pattern was observed for **NF-9** and **NF-7** which was around 3.5 folds and 2 folds more active than **NF-8**. Overall the preference order for positioning of the substituents follows the order: *para* > *ortho* > *meta*. It was also observed that the nature of substituents at *p*-position influenced the activity as substituents with a stronger $-I$ (Inductive) effect such as fluoro, nitro and bromo remarkably favoring the inhibitory potential in comparison to the substituents with stronger $+R$ (Resonance) (OCH_3) and hyper conjugation effect (CH_3) (Compare **NF-2**, **4**, **12** with **NF-14**, **15**). The remarkable inhibitory potential of **NF-4** possessing fluoro substituted (*para*) phenyl ring could be attributed to the presence of fluorophilic environments in the cavity of enzyme. In general, F substituents on ligands prefer to orient toward electropositive regions of receptor sites[21] and this has led to the increased use of this element to enhance the binding affinity to the target protein.[22] Moreover the ability of fluorine to get involved in hydrogen bonding/dipolar interactions have also been evidenced through number of reports.[22,23] Thus fluorine effect could be the probable reason for the significant inhibition of xanthine oxidase by **NF-4**.

Table 2 represents the inhibitory potential of **NF-2**, **4**, **9**, **12** (displaying IC_{50} values <10 μM) at five different concentration.

Figure 2 represents the % age inhibition of potent naphthoflavones against xanthine oxidase.

Figure 3 represents the plot of V versus [I] for the most potent inhibitor, that is, **NF-4**.

Enzyme kinetic study was also studied for the potent compounds. The Lineweaver–Burk plot (Fig. 4) revealed that compounds **NF-2**, **NF-4**, **NF-12** were mixed-type XO inhibitors. The pattern of graph shows that it is a form of mixed inhibition scenario. The K_m, V_{max} and slope are all affected by the inhibitor. The inhibitors have increased the K_m and slope (K_m/V_{max}) while decreasing the V_{max}. Moreover carefully observing the Figure 4, it was found that intersecting lines on the graph converge to the left of the *y*-axis and above the *x*-axis which indicates that the value of α (a constant that defines the degree to which inhibitor binding affects the affinity of the enzyme for substrate) is greater than 1.[24] This confirms that the inhibitor preferentially binds to the free enzyme and not the enzyme substrate complex. Therefore, the mode of inhibition of **NF-2**, **NF-4** and **NF-12** is mixed-type but it seems that they have a strong competitive component.

In order to understand the binding conformation of **NF-4** (most potent XO inhibitor), it was docked using the GOLD software.[25] into the salicylic acid binding site of XO.[26]

To validate the docking procedure, the salicylic acid was extracted from the original X-ray structure of XO (1FIQ)[26] and docked using GOLD. The highest scoring conformation was selected and compared with original X-ray structure conformation. The docked conformation of salicylic acid was found to be the similar with the original X-ray structure. The root mean square deviation (RMSD) between the best scored conformation from docking and X-ray structure was found to be 0.21 Å.

The best fit conformation of **NF-4** was selected on the basis of Chemplp scoring function and visual inspection. The Figure 5 shows the binding conformation of the **NF-4** at the binding site of XO. The binding site residues and overall binding mode was found to be similar to those observed with salicylic acid[26] and fabuxostat.[27] The **NF-4** gets stabilized by various electrostatic and hydrophobic interactions. In docking pose, the naphthyl ring (ring A and B) of **NF-4** was found sandwiched between Phe914 and Phe1009. The Phe914 and Phe1009 are involved in 'face-to-face' and 'face-to-edge' pai–pai stacking interactions, respectively with **NF-4**. This arrangement of energetically favorable arene/arene interactions[28] is also present in the co-crystal structure of XO with salicylate[23] and fabuxostat.[27] Its conservation argues for an important role in stabilizing the binding positions of aromatic inhibitors and also represent one of the key features of substrate recognition (Fig. 5).[7] Two crucial H-bond interactions were observed between **NF-4** and binding residues of XO. Here, first H-bond was formed between hydroxyl group of Ser876 and carbonyl group of ring C (d = 1.91 Å; θ = 145.9°). Also, the fluorine atom at ring D is involved in H-bond interaction with side chain N–H of Asn768 (d = 2.24 Å; θ = 101.1°). This can be suggested that these H-bonds may contribute significantly towards the binding free energy between **NF-4** and XO. The ring D of **NF-4** face towards the side chain of Leu648 and showed the hydrophobic interaction (Fig. 5).

The present study employs molecular hybridization technique for the design of naphthoflavones as hybrids of naphthopyrans and flavones (two previously reported classes of xanthine oxidase

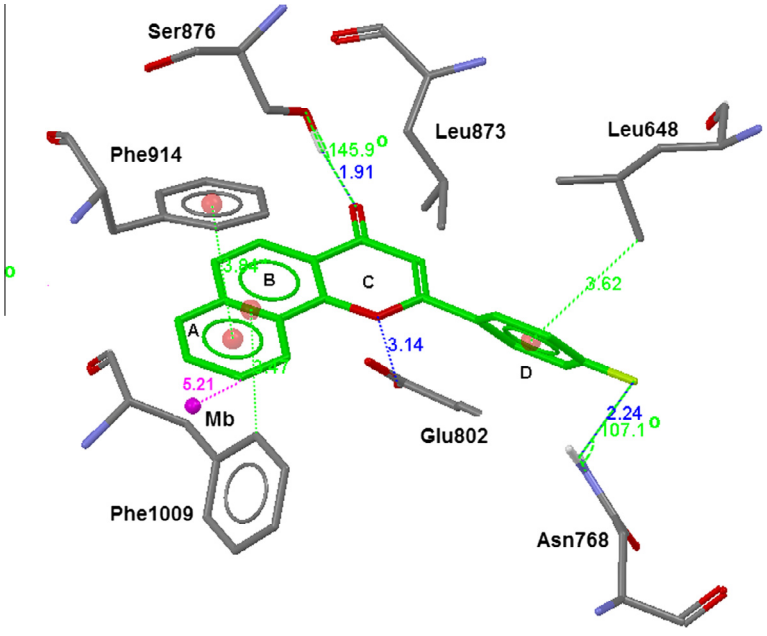


Figure 5. Binding interactions of **NF-4** with the amino acid residues.

inhibitors). The designed hybrids have been synthesized and evaluated for xanthine oxidase inhibition for the first time. Structural features such as substituent free C-3 and overall planarity were kept intact as revealed by the well established structure activity relationship of flavones. Naphthopyran was employed as a surrogate for the hydroxy substituted benzopyran in flavone framework. In vitro xanthine oxidase inhibitory assay indicated the promising inhibitory profile of the naphthoflavones as the most potent naphthoflavones, that is, **NF-4** and **NF-12** displayed strong inhibition at 0.62 μM and 1.95 μM. Structure activity relationship indicated that halo and nitro at *para* position of phenyl ring (2nd position) were the preferred substituents for potent inhibition. Naphthoflavones were found to be mixed type inhibitors as both the K_m and V_{max} were changed with the most potent naphthoflavones and the intersection points of the plot indicated strong competitive component of the inhibitors. Molecular modeling study confirmed naphthopyran as a potential surrogate owing to its interactions with the amino acid residues. **NF-4** displayed interesting interactions with the amino acid residues in the substrate binding site further supporting the competitive aspect of the mixed type inhibitor. Thus **NF-4** and **NF-12** appears to the good hits among the series of naphthoflavones and displays promising attributes for detailed investigation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.07.041.

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18. *2-(2-Fluorophenyl)-4H-benzo[h]chromen-4-one (**NF-2**)*: Yield: 71%; mp: 118–120 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ, TMS = 0): 8.55 (1H, m), 8.17 (1H, d, J = 8.7 Hz), 7.92–8.05 (2H, m), 7.69–7.79 (3H, m), 7.55 (1H, m), 7.24–7.41 (2H, m), 6.91 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ, TMS = 0): 113.2, 113.4, 117.0, 117.3, 120.1, 120.4, 120.7, 122.4, 124.0, 124.4, 124.8, 125.4, 127.2, 128.2, 129.0, 129.3, 132.8, 133.0, 136.0, 153.7, 158.3, 158.8, 162.2, 178.2. Anal. Calcd for $C_{19}H_{11}FO_2$: C, 78.61; H, 3.82; F, 6.54. Found: C, 78.73; H, 3.54. *2-(4-Fluorophenyl)-4H-benzo[h]chromen-4-one (**NF-4**)*: Yield: 78%; mp: 154–156 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ, TMS = 0): 8.55 (1H, br s), 8.14 (1H, d, J = 8.7 Mz), 7.92–8.07 (3H, m), 7.77 (1H, d, J = 8.7 Hz), 7.64–7.74 (2H, m), 7.23–7.29 (2H, m), 6.91 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ, TMS = 0): 108.5, 116.3, 116.6, 120.0, 120.6, 122.2, 124.0, 125.5, 127.2, 128.0, 128.3, 128.4, 128.5, 129.3, 136.0, 153.5, 161.8, 178.1. Anal. Calcd for $C_{19}H_{11}FO_2$: C, 78.61; H, 3.82; F, 6.54. Found: C, 79.00; H, 3.53. *2-(4-Nitrophenyl)-4H-benzo[h]chromen-4-one (**NF-12**)*: Yield: 76%; mp: 73–75 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ, TMS = 0): 8.60 (1H, m), 8.45 (2H, d, J = 9.00 Hz), 8.18 (3H, m), 8.01 (1H, m), 7.67–7.86 (3H, m), 7.08 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ, TMS = 0): 110.8, 120.3, 120.5, 122.2, 124.4, 126.0, 127.1, 127.5, 128.4, 129.7, 137.7, 160.0, 177.9. MS: m/z: 318 (M^++1); Anal. Calcd for $C_{19}H_{11}NO_4$: C, 71.92; H, 3.49; N, 4.41. Found: C, 71.65; H, 3.15; N, 4.75.
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