European Journal of Medicinal Chemistry 45 (2010) 2345–2352

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and cyclooxygenase inhibitory activities of some *N*-acylhydrazone derivatives of isoxazolo[4,5-d]pyridazin-4(5*H*)-ones

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ARTICLE INFO

Article history:
Received 3 January 2010
Received in revised form
3 February 2010
Accepted 4 February 2010
Available online 10 February 2010

Keywords:
Cyclooxygenase enzymes
Isoxazolo[4,5-d]pyridazin-4(5*H*)-one
N-Acylhydrazone
Docking

ABSTRACT

In this study, new isoxazolo[4,5-d]pyridazin-4(5*H*)-one derivatives having an *N*-acylhydrazone moiety were synthesized. The compounds were tested for their COX inhibitory activities using NS-398 and indomethacine as reference compounds. Although the compounds had an inhibitory profile against both COX-1 and COX-2, most were found to be more selective against COX-2 by a small percentage of inhibition, at the concentration of 50 μM. Docking studies were done to understand the interactions of the tested compounds with the active site of COX-2. It was observed that the compounds fit into, and interacted with, the hydrophobic parts which are common in the active pocket of COX-1 and COX-2 enzymes but could not fit to the area which is specific for COX-2 enzyme.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutics for the treatment of pain and inflammation. The first NSAID discovered was aspirin which has now been used for more than 100 years. However, the molecular mechanism of aspirin and other NSAIDs was elucidated only in the 1970s. Vane [1] proposed that NSAIDs produce their therapeutic and also unwanted effects by inhibition of cyclooxygenase (COX) enzyme in prostaglandin synthetic pathway. Until recently, all prostaglandin synthesis was thought to result from only one form of COX enzyme. In the early 1990s it was discovered that COX exists in two isoforms, COX-1 (constitutive form) and COX-2 (inducible form). Thus a new hypothesis has been suggested to explain the effects of NSAIDs; inhibition of COX-1 accounts for the undesirable side effects, whilst inhibition of COX-2 accounts for the therapeutic benefits of NSAIDs. This proposal has led to drug discovery programs aimed at identifying new anti-inflammatory agents that selectively inhibit COX-2 activity, and a large number of compounds with common structural features, i.e., the presence of two aryl rings on adjacent carbons of a cyclic/acylic moiety, has been investigated for COX-2 inhibition. Among these compounds, rofecoxib, celecoxib, valdecoxib and etoricoxib were approved and marketed. However, rofecoxib was banned in 2004 because of cardiac toxicity. Subsequently, some of the other coxibs have been voluntarily withdrawn from the market [2]. On the other hand, some studies have suggested that rofecoxib's adverse cardiac events may not be a class effect but rather an intrinsic chemical property related to its metabolism [3].

For this reason novel scaffolds with COX-2 selective inhibitory activity needed to be found and evaluated for their anti-inflammatory effects. A number of molecules based upon a monocyclic (5 or 6 membered) or bicyclic heterocyclic template have been investigated for their COX-2 inhibitory activity. Among them, some compounds having a pyridazinone structure (Fig. 1) have showed selective COX-2 inhibitory activity [4–6]. Furthermore, the analgesic and anti-inflammatory effects of *N*-acylhydrazones substituted with different structures have been described [7–14].

These observations prompted us to synthesize some new 7-benzyl-3-methylisoxazolo[4,5-d]pyridazin-4(5*H*)-one derivatives, bearing an *N*-acylhydrazone moiety at the fifth position, as potential COX-2 inhibitors.

2. Results and discussion

2.1. Chemistry

The synthetic route used to synthesize *N*′-substitutedbenzylidene-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5*H*)-yl)acetohydrazides (**7**–**28**) is outlined in Scheme 1. 7-Benzyl-3-

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doi:10.1016/j.ejmech.2010.02.012

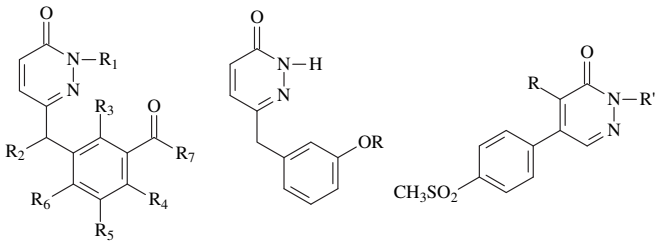


Fig. 1. Selective COX-2 inhibitors having pyridazinone structure.

methylisoxazolo[4,5-d]pyridazin-4(5*H*)-one **4** was prepared using the method which was described earlier [15,16]. But we observed that using methyl acetoacetate instead of ethyl acetoacetate increased the reaction yield of **1**.

Compound **4** was transformed into ethyl 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5*H*)yl)acetate **5** by reacting with ethyl bromoacetate in the presence of potassium carbonate. By refluxing this compound with excess hydrazine hydrate, 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5*H*)-yl)acetohydrazide **6** was obtained. Finally, the reaction of this hydrazide with a number of benzaldehydes in the presence of hydrochloric acid gave **7**–**28**.

Structures of the target compounds **7**–**28**, were elucidated by using spectral methods (IR, ^{1}H NMR, ESI-MS) and elemental analysis. In the IR spectra of **7**–**28**, we observed the bands at around 1700 cm^{-1} and 1670 cm^{-1} due to C=O (pyridazinone) and C=O (hydrazide) stretching respectively. In addition to these bands, a broad band appeared between 3600 and 3270 cm^{-1} due to (CO)N–H stretching.

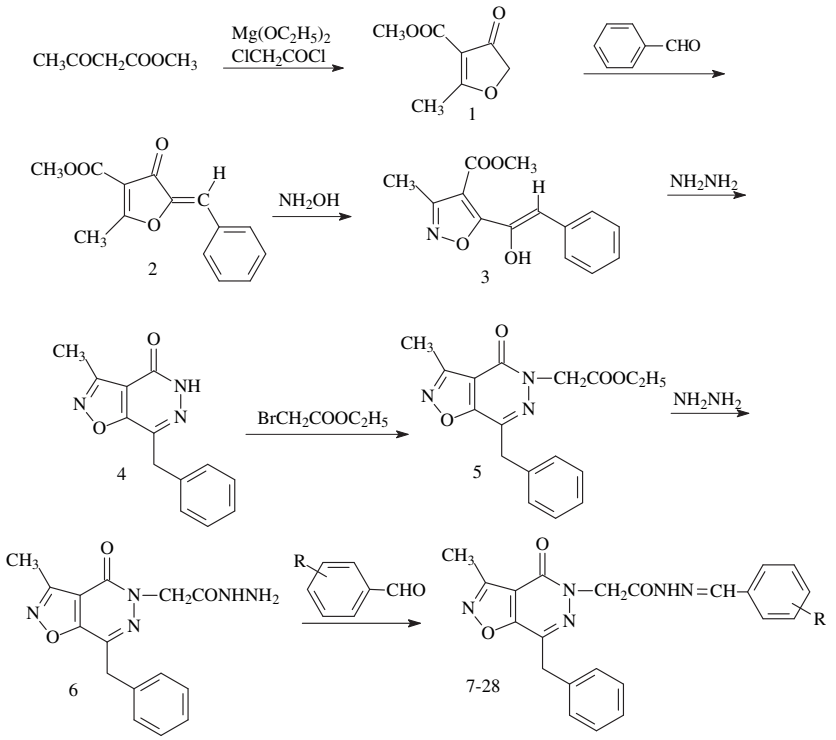
It is known that *N*-acylhydrazones may exist as geometrical isomers (*E*/*Z*) in respect to the C=N double bonds and as rotamers (*cis*/*trans*) about amide N–C(O) bond [17,18]. Palla and co-workers [17] showed that *N*-acylhydrazones derived from aromatic aldehydes in solution are in the *E* form because of the hindered rotation on the imine bond.

Syakaev and co-workers [18], who investigated the ^{1}H NMR spectra of *N*-acylhydrazones derived from acetone and aromatic aldehydes, mentioned that *N*-acylhydrazone derived from acetone had two rotamers due to the N–C(O) bond because of symmetry around the C=N bond. They also noticed that, *N*-acylhydrazones derived from acetone and aromatic aldehydes demonstrated a similar pattern of ^{1}H NMR spectra. By regarding these data, they claimed that *N*-acylhydrazones derived from aromatic aldehydes have two rotamers (*cis* and *trans*) due to the N–C(O) bond.

In our study, to understand the possible isomers of **7**–**28**, we calculated the relative free energy of eight isomers of **7** derived from 2-fold rotations about the C(O)–N, N–N and C=N bonds. These calculations were performed at an ab initio level of theory using the electronic structure code, Gaussian 03. According to the optimization studies, it was observed that the two most stable isomers are *E*/*E*/*trans* and *E*/*E*/*cis* conformers (Fig. 2), which is consistent with the literature.

In the ^{1}H NMR spectra of **7**–**28**, we observed the signals belonging to N=CH, –CH$_2$–C(O) and N–H protons (also some protons in benzylidene) as double singlets. By regarding both literature data and geometric optimization results we assumed that these double singlets appeared because of *cis* and *trans* isomers of **7**–**28**. The signals belonging to methylidene and imine protons were seen at around 4.80 (*trans*), 5.20 (*cis*) ppm and 8.30 (*trans*), 7.90 (*cis*) ppm, respectively. The amide protons also appeared at around 11–12 ppm as double singlets with some exception.

In the ESI mass spectra of **7**–**28**, in addition to $[M + Na]^+$, the fragment formed by the cleavage of N–N bond with simultaneous hydrogen migration was seen at *m*/*z* 282, in accord with literature data [19,20].



Scheme 1. Synthetic route of **1**–**28**.

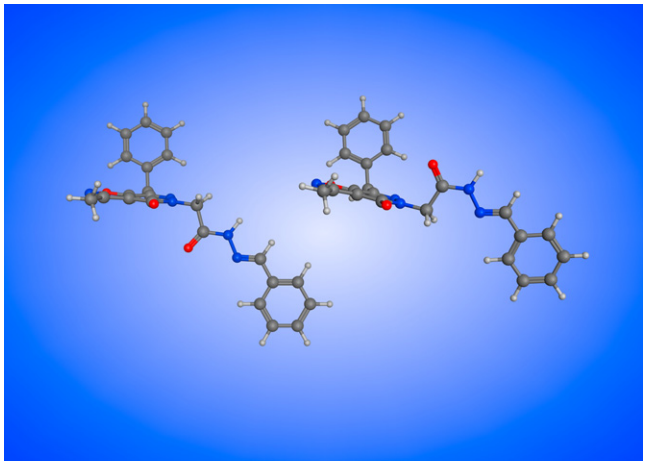


Fig. 2. Structure of the *E*/*E*/*trans* (left) and *E*/*E*/*cis* (right) conformers of **7**.

2.2. Pharmacology

The inhibitory effects of the target compounds (**7**–**28**) on COX-1 and COX-2 enzymes were evaluated at 50 μM using a COX Inhibitor Screening Kit. NS-398 and indomethacine were used as reference compounds. The results are shown in Table 1. Although the target compounds showed remarkable inhibitory activity on COX-2 enzyme, none of them was found as effective as the standard compound, NS-398. Furthermore, when their activities were compared with indomethacine, it was determined that all the compounds inhibited COX-2 enzyme more than indomethacine while, with the exception of **15**, they inhibited COX-1 enzyme less than that.

The compound carrying a fluoro substituent at the third position of the phenyl ring, **15**, appeared as the most active compound in this series against COX-2 enzyme. In addition, its inhibitory activity on the COX-1 enzyme was found to be higher even than that of indomethacine. Among the series, the compounds having chloro and fluoro substituents at the second position of the phenyl ring (**11** and **14** respectively) were the most notable ones because they showed a remarkable inhibitory activity on COX-2 enzyme with inhibiting COX-1 enzyme less than indomethacine (Table 1).

Table 1
In vitro COX-1 and COX-2 enzyme inhibition data for **7**–**28**.

Compound	R	% Inhibition (50 μM)[a]	
		COX-1	COX-2
7	H	50.6 ± 2.9	52.0 ± 4.5
8	2-Br	52.8 ± 4.0	50.1 ± 2.7
9	3-Br	42.9 ± 1.3	53.9 ± 3.5
10	4-Br	50.6 ± 6.2	53.9 ± 1.7
11	2-Cl	52.8 ± 4.0	65.5 ± 1.6
12	3-Cl	36.3 ± 3.7	57.8 ± 4.3
13	4-Cl	39.6 ± 3.1	55.6 ± 3.6
14	2-F	41.8 ± 4.8	64.8 ± 3.4
15	3-F	83.0 ± 1.5	71.7 ± 2.6
16	4-F	54.0 ± 2.1	59.7 ± 5.2
17	2-CH_3	46.9 ± 3.6	56.0 ± 3.8
18	3-CH_3	61.0 ± 1.4	54.3 ± 2.8
19	4-CH_3	62.1 ± 5.0	57.8 ± 4.1
20	2-CF_3	51.0 ± 4.3	45.5 ± 3.7
21	3-CF_3	47.1 ± 1.9	35.0 ± 4.4
22	4-CF_3	64.0 ± 5.2	49.0 ± 3.9
23	2-OCH_3	65.0 ± 2.4	49.0 ± 5.3
24	3-OCH_3	55.2 ± 4.2	59.5 ± 2.6
25	4-OCH_3	46.3 ± 3.1	59.4 ± 2.1
26	2-NO_2	59.0 ± 2.7	57.8 ± 4.7
27	3-NO_2	39.6 ± 3.9	55.8 ± 3.3
28	4-NO_2	40.7 ± 2.2	49.0 ± 4.1
Indomethazine		78.0 ± 2.4	29.6 ± 2.3
NS-398		–	91.1 ± 3.0

[a] The determination was performed in duplicate for two independent experiments.

In addition to these data, we observed that the substitution of triflouromethyl group to the third position of the phenyl ring (**21**) resulted in a remarkable decrease in COX-2 inhibitory potency, whereas a Cl, Br, or NO_2 group showed good selectivity for COX-2 over COX-1.

Furthermore, we noticed that the substituents on the phenyl ring (except **15** and **21**) did not cause an important effect on the activity.

The docking study was performed with MOE software program to understand the orientations of the ligands in COX-2. To compare orientations of the ligands, we superimposed each compound's best pose which was obtained by regarding the S score in MOE. Except **21** (3-CF_3), **24** (3-OCH_3) and **27** (3-NO_2), all the ligands showed similar orientation in the COX-2 active site (Fig. 3A). It was observed that the benzyl ring of the compounds fitted into the cavity formed by Phe381, Leu384, Tyr385, Trp387, Phe513 and Ser530 while the 3-methylisoxazole moiety fitted into the other cavity formed by Met113, Val116, Val349, Tyr355, Leu359 and Leu531 (Fig. 3B). As Kurumbail and co-workers [21] mentioned, these features are common in non-selective and selective COX-2 inhibitors such as flurbiprofen, indomethacine and SC-558. Furthermore, the OH group of Tyr355 formed a hydrogen bond (∼2.1 Å) with the C=O moiety of the pyridazinone, and the C=O moiety of Leu352 and N–H moiety of His90 hydrogen bonded to the NH and C=N of the hydrazone respectively (Fig. 3C).

On the other hand, it is known that the main difference between the two COX active sites is the replacement of Ile523 in COX-1 by Val, a less bulky amino acid. This replacement creates an adjunct pocket in the COX-2 active site which may be responsible for selectivity. Our docking study showed that **7**–**28** were oriented so that their *N*-acylhydrazone moiety did not fill this adjunct pocket (Fig. 3B). This may explain why the compounds did not show significant selective activity against COX-2 enzyme.

Additionally, **21**, which showed the lowest inhibitor activity against COX-2 enzyme, oriented in a different way altogether (Fig. 3D). This suggests that the general orientation obtained for the other compounds was correct.

3. Conclusions

In the present study, a series of new *N*′-substitutedbenzylidene-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5*H*)-yl)acetohydrazide derivatives were synthesized in order to evaluate their inhibitory activities on COX-1 and COX-2 enzymes. The binding mode of the tested compounds inside the COX-2 active site was predicted using a docking technique. When the results of the inhibitory activity and docking studies are considered together, it can be suggested that: (i) the isoxazolo[4,5-d]pyridazin-4(5*H*)-one moiety is a suitable scaffold for COX-2 enzyme; (ii) the *N*-acylhydrazone moiety, which does not fill the adjunct pocket in the COX-2 active site, does not make any contribution to the selectivity; (iii) instead of *N*-acylhydrazone, appropriate substitutions which can fill the adjunct pocket and interact with the relatively polar residues such as Gln192 and Arg513 may be useful to propose new molecules with enhanced selectivity towards COX-2.

4. Experimental

4.1. Chemistry

Melting points were determined with a Thomas-Hoover Capillary Melting Point Apparatus (Philadelphia, PA, USA) and are

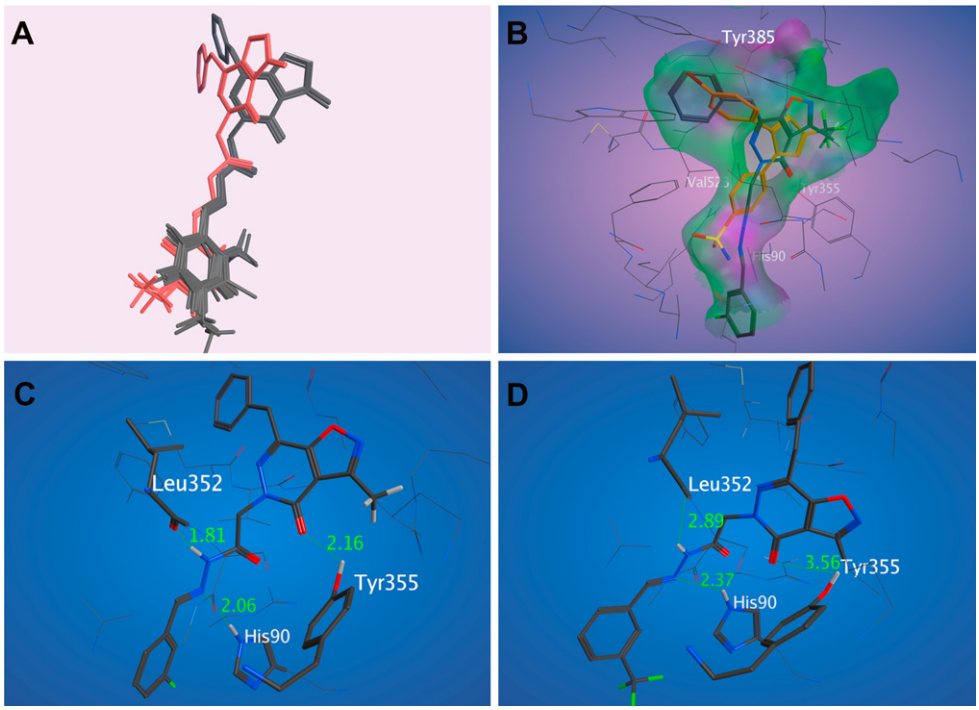


Fig. 3. (A) Superimposition of best poses of **7–28** (**21**, **24** and **27** are shown as pink). (B) The orientation of **15** in COX-2 active pocket (SC-558 is shown as orange). (C) The orientation of **15** in COX-2 active pocket (H bonds are shown as green). (D) The orientation of **21** in COX-2 active pocket (H bonds are shown as green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

uncorrected. IR spectra (KBr) were recorded on a Jasco FTIR420 Fourier spectrometer and were reported in cm^{-1}. The ^1H NMR spectra (DMSO-d_6) were recorded on a Varian Mercury 400 FT NMR spectrophotometer using TMS as an internal reference (chemical shift represented in δ ppm). The ESI-MS spectra were measured on a micromass ZQ-4000 single quadruple mass spectrometer. Elemental analyses (C, H, N) were performed on Leco CHNS 932 analyzer.

4.1.1. Methyl 2-methyl-4-oxo-4,5-dihydrofuran-3-carboxylate (**1**) [15]

Methyl acetoacetate (0.020 mol) was added to the suspension of magnesium ethoxide (0.022 mol) in 6 mL absolute ethanol and 25 mL toluene, then the mixture was stirred at room temperature for 1 h. The mixture was cooled to −10 °C followed by addition of acetonitrile (25 mL). The chloroacetyl chloride (0.022 mol) was added dropwise to the stirred solution which was then kept at 0 °C for 1 h. Then the mixture was poured into ice/water (65 mL) containing sulfuric acid (4 mL) and organic phase extracted with ether, separated and dried (Na_2SO_4). Triethylamine (0.020 mol) in dry ether (5 mL) was added to cooled solution (0 °C). The precipitated salt was filtered off and the filtrate was evaporated. The residue was recrystallized from petroleum ether (45% yield). Mp 110–1 °C. IR: 1711 (C=0, ester and ring). ^1H NMR: 2.50 (3H, s, CH_3), 3.63 (3H, s, $-OCH_3$), 4.72 (2H, s, $-CH_2-$). ESI-MS (*m*/*z*); 179 $[M + Na]^+$ (100%), 157 $[M + H]^+$.

4.1.2. Methyl 5-benzylidene-2-methyl-4-oxo-4,5-dihydrofuran-3-carboxylate (**2**) [15]

Compound **1** (3.25 mmol) and equimolar amount of benzaldehyde were dissolved in 25 mL of anhydrous benzene. *p*-Toluenesulfonic acid (0.08 g) was added as a catalytic reagent. The mixture was then heated with a Dean–Stark separator during 2 h. Benzene was evaporated and residue recrystallized from ethyl acetate and *n*-hexane mixture (1:1) (64% yield). Mp 118–9 °C. IR (KBr); 1707 (C=O, ester), 1655 (C=O, ring). ^1H NMR (DMSO-d_6, 400 MHz); δ 2.75 (3H, s, $-CH_3$), 3.76 (3H, s, $-OCH_3$), 6.90 (1H, s, –CH=), 7.5–7.52 (3H, m, phenyl-H_3, H_4, H_5), 7.92–7.95 (2H, m, phenyl–H_2, H_6). ESI-MS (*m*/*z*); 267 $[M + Na]^+$ (100%), 245 $[M + H]^+$.

4.1.3. Methyl 5-(1-hydroxy-2-phenylvinyl)-3-methylisoxazole-4-carboxylate (**3**) [16]

A mixture of **2** (0.01 mol), sodium acetate (0.012 mol), hydroxylamine hydrochloride (0.011 mol), water (10 mL) and ethanol (50 mL) was refluxed for 2 h. After cooling, yellow crystals were collected and recrystallized from ethanol to give pure compound (66% yield). Mp 135–6 °C. IR (KBr); 1672 (C=O). ^1H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H, s, $-CH_3$), 3.85 (3H, s, $-OCH_3$), 6.27 (1H, s, –CH=), 7.33–7.39 (3H, m, phenyl–H_3, H_4, H_5), 7.72–7.82 (2H, m, phenyl–H_2, H_6), 10.53 (1H, s, –OH). ESI-MS (*m*/*z*); 282 $[M + Na]^+$ (100%), 260 $[M + H]^+$.

4.1.4. 7-Benzyl-3-methylisoxazolo[4,5-d]pyridazin-4(5H)-one (**4**) [16]

This compound was prepared according to the literature method [16].

4.1.5. Ethyl 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetate (**5**)

Anhydrous potassium carbonate (0.075 mol) was added to a solution of 0.05 mol **4** in acetone (100 mL). The reaction mixture was refluxed for 4 h and then to it was added drop by drop a solution of 0.05 mol ethyl bromoacetate in acetone (25 mL). The reaction mixture was refluxed for 2 h and poured into ice-cold water (250 mL). The solid was filtered and purified by recrystallization from ethanol to give **5** (98% yield). Mp 135–6 °C. IR (KBr); 1745 (C=O, ester), 1694 (C=O, pyridazinone). ^1H NMR (DMSO-d_6, 400 MHz); δ 1.20 (3H, t, $-CH_2-CH_3$), 2.53 (3H, s, $-CH_3$), 4.16 (2H, q, $-CH_2-CH_3$), 4.23 (2H, s, $-CH_2-C_6H_5$), 4.97 (2H, s, $-CH_2COO-$), 7.24–7.34 (5H, m, phenyl). ESI-MS (*m*/*z*); 350 $[M + Na]^+$ (100%), 271, 185.

Anal. Calcd. for $C_{17}H_{17}N_3O_4$: C, 62.38; H, 5.23; N, 12.84. Found: C, 62.21; H, 5.09; N, 12.89.

*4.1.6. 2-(7-Benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**6**)*

A solution of 0.20 mol of hydrazine hydrate (100%) and 0.01 mol of **5** in 50 mL of absolute ethanol was refluxed with stirring for 1 h. The solvent was evaporated under reduced pressure. The residue was purified by recrystallization from ethanol to give **6** (90% yield). Mp 191–2 °C. IR (KBr); 3341, 3263 (N–H), 1687 (C=O, pyridazinone), 1664 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.52 (3H, s, –CH_3), 4.20 (2H, s, –CH_2–C_6H_5), 4.29 (2H, br, –NH_2), 4.74 (2H, s, –CH_2–CONH–), 7.20–7.38 (5H, m, phenyl), 9.25 (1H, br, –NH–). ESI-MS (*m*/*z*); 336 $[M + Na]^+$ (100%), 286, 240. Anal. Calcd. for $C_{15}H_{15}N_5O_3$: C, 57.50; H, 4.83; N, 22.35. Found: C, 57.57; H, 4.86; N, 22.18.

*4.1.7. General procedure for the preparation of N-acylhydrazones (**7**–**28**)*

An equimolar amount of appropriate aromatic aldehyde was added to a solution of hydrazide (**6**) in 20 mL of ethanol, in presence of catalytic amount of HCl (2 drops). Reaction mixture was stirred for 0.5–2 h at room temperature and poured into cold water. After neutralization with 10% aqueous sodium bicarbonate solution, the precipitate formed was filtered, washed three times with 20 mL water and purified by recrystallization from acetonitrile.

*4.1.7.1. N′-Benzylidene-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]-pyridazin-4(5H)-yl)acetohydrazide (**7**).* The derivative **7** was obtained as a white solid by condensation of **6** with benzaldehyde in 80% yield, mp 253–4 °C. IR (KBr); 3550–3300 (N–H), 1700 (C=O, pyridazinone), 1671 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.53 (3H; s; –CH_3), 4.23 (2H; s; –CH_2–C_6H_5), 4.92 and 5.32 (2H; s; –CH_2–CONH–), 7.23–7.31 (5H; m; –CH_2–C_6H_5), 7.42–7.50 (3H; m; –N=CH–C_6H_5, *H*$_3$, *H*$_4$, *H*$_5$), 7.69–7.73 (2H; m; –N=CH–C_6H_5, *H*$_2$, *H*$_6$), 8.03 and 8.21 (1H; s; –N=CH–), 11.78 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 424 $[M + Na]^+$ (100%), 282, 219, 132. Anal. Calcd. for $C_{22}H_{19}N_5O_3$: C, 65.83; H, 4.77; N, 17.45. Found: C, 65.76; H, 4.61; N, 17.36.

*4.1.7.2. N′-(2-Bromobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**8**).* The derivative **8** was obtained as a white solid by condensation of **6** with 2-bromobenzaldehyde in 97% yield, mp 183–4 °C. IR (KBr); 3620–3360 (N–H), 1690 (C=O, pyridazinone and hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; –CH_3), 4.10 (2H; s; –CH_2–C_6H_5) 4.80 and 5.21 (2H; s; –CH_2–CONH–), 7.08–7.32 (7H; m; –CH_2–C_6H_5, –N=CH–C_6H_5, *H*$_4$, *H*$_5$), 7.55 (1H; d; –N=CH–C_6H_5, *H*$_3$, *J*: 8.0 Hz), 7.78 and 7.87 (1H; d; –N=CH–C_6H_5, *H*$_6$, *J*: 8.0 Hz), 8.24 and 8.42 (1H; s; –N=CH–), 11.84 and 11.90 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 504 $[M + Na + 2]^+$, 502 $[M + Na]^+$ (100%), 282, 136. Anal. Calcd. for $C_{22}H_{18}BrN_5O_3$: C, 55.01; H, 3.78; N, 14.58. Found: C, 54.92; H, 3.99; N, 14.54.

*4.1.7.3. N′-(3-Bromobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**9**).* The derivative **9** was obtained as a white solid by condensation of **6** with 3-bromobenzaldehyde in 90% yield, mp 239–40 °C. IR (KBr); 3640–3360 (N–H), 1698 (C=O, pyridazinone), 1673 (C=O, hydrazide), ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; –CH_3), 4.09 (2H; s; –CH_2–C_6H_5), 4.81 and 5.20 (2H; s; –CH_2–CONH–), 7.09–7.18 (5H; m; –CH_2–C_6H_5), 7.23–7.27(1H; m; –N=CH–C_6H_5, *H*$_5$), 7.46 (1H; d; –N=CH–C_6H_5, *H*$_4$, *J*: 7.2 Hz), 7.57 (1H; d; –N=CH–C_6H_5, *H*$_6$, *J*: 7.2 Hz), 7.76 and 7.80 (1H; s; –N=CH–C_6H_5, *H*$_2$), 7.86 and 8.05 (1H; s; –N=CH–), 11.75 and 11.78 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 504 $[M + Na + 2]^+$, 502 $[M + Na]^+$ (100%), 282. Anal. Calcd. for $C_{22}H_{18}BrN_5O_3$: C, 55.01; H, 3.78; N, 14.58. Found: C, 54.89; H, 3.73; N, 14.50.

*4.1.7.4. N′-(4-Bromobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**10**).* The derivative **10** was obtained as a white solid by condensation of **6** with 4-bromobenzaldehyde in 87% yield, mp 264 °C. IR (KBr); 3670–3380 (N–H), 1699 (C=O, pyridazinone), 1670 (C=O, hydrazide), ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.39 (3H; s; –CH_3), 4.09 (2H; s; –CH_2–C_6H_5), 4.79 and 5.18 (2H; s; –CH_2–CONH–), 7.10–7.18 (5H; m; –CH_2–C_6H_5), 7.48–7.55 (4H; m; –N=CH–C_6H_5, *H*$_2$, *H*$_3$, *H*$_5$, *H*$_6$), 7.87 and 8.05 (1H; s; –N=CH–), 11.70 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 504 $[M + Na + 2]^+$, 502 $[M + Na]^+$ (100%), 282, 239. Anal. Calcd. for $C_{22}H_{18}BrN_5O_3$: C, 55.01; H, 3.78; N, 14.58. Found: C, 54.86; H, 3.77; N, 14.46.

*4.1.7.5. N′-(2-Chlorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**11**).* The derivative **11** was obtained as a white solid by condensation of **6** with 2-chlorobenzaldehyde in 93% yield, mp 191–2 °C. IR (KBr); 3620–3320 (N–H), 1700 (C=O, pyridazinone), 1671 (C=O, hydrazide), ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; –CH_3), 4.10 (2H; s; –CH_2–C_6H_5), 4.80 and 5.21 (2H; s; –CH_2–CONH–), 7.09–7.20 (8H; m; –CH_2–C_6H_5, –N=CH–C_6H_5, *H*$_3$, *H*$_4$, *H*$_5$), 7.80 and 7.89 (1H; dd; –N=CH–C_6H_5, *H*$_6$, *J*: 7.6 Hz, *J*: 1.6 Hz), 8.28 and 8.47 (1H; s; –N=CH–), 11.82 and 11.87 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 460 $[M + Na + 2]^+$, 458 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}ClN_5O_3$: C, 60.62; H, 4.16; N, 16.07. Found: C, 60.55; H, 4.32; N, 16.05.

*4.1.7.6. N′-(3-Chlorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**12**).* The derivative **12** was obtained as a white solid by condensation of **6** with 3-chlorobenzaldehyde in 87% yield, mp 243 °C. IR (KBr); 3640–3310 (N–H), 1698 (C=O, pyridazinone), 1673 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; –CH_3), 4.09 (2H; s; –CH_2–C_6H_5), 4.80 and 5.20 (2H; s; –CH_2–CONH–), 7.09–7.20 (5H; m; –CH_2–C_6H_5), 7.31–7.35 (2H; m; –N=CH–C_6H_5, *H*$_4$, *H*$_5$), 7.53 (1H; d; –N=CH–C_6H_5, *H*$_6$, *J*: 4.8 Hz), 7.62 and 7.67 (1H; s; –N=CH–C_6H_5, *H*$_2$), 7.87 and 8.06 (1H; s; –N=CH–), 11.75 and 11.77 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 460 $[M + Na + 2]^+$, 458 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}ClN_5O_3$: C, 60.62; H, 4.16; N, 16.07. Found: C, 60.76; H, 4.15; N, 16.05.

*4.1.7.7. N′-(4-Chlorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**13**).* The derivative **13** was obtained as a white solid by condensation of **6** with 4-chlorobenzaldehyde in 62% yield, mp 266 °C. IR (KBr); 3650–3310 (N–H), 1699 (C=O, pyridazinone), 1671 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.54 (3H; s; –CH_3), 4.24 (2H; s; –CH_2–C_6H_5), 4.95 and 5.34 (2H; s; –CH_2–CONH), 7.24–7.35 (5H; m; –CH_2–C_6H_5), 7.50–7.53 (2H; m; –N=CH–C_6H_5, *H*$_3$, *H*$_5$), 7.73–7.77 (2H; m; –N=CH–C_6H_5, *H*$_2$, *H*$_6$), 8.03 and 8.21 (1H; s; –N=CH–), 11.86 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 460 $[M + Na + 2]^+$, 458 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}ClN_5O_3$: C, 60.62; H, 4.16; N, 16.07. Found: C, 60.48; H, 3.95; N, 15.98.

*4.1.7.8. N′-(2-Fluorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**14**).* The derivative **14** was obtained as a white solid by condensation of **6** with 2-fluorobenzaldehyde in 93% yield, mp 212–3 °C. IR (KBr); 3680–3330 (N–H), 1699 (C=O, pyridazinone), 1673 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; –CH_3), 4.09 (2H; s; –CH_2–C_6H_5), 4.79 and 5.20 (2H; s; –CH_2–CO–), 7.08–7.20 (7H; m; –CH_2–C_6H_5, –N=CH–C_6H_5, *H*$_3$, *H*$_5$),

7.31–7.36 (1H; m; –N=CH–C_6H_5, *H4*), 7.73–7.53 and 7.79–7.83 (1H; m; –N=CH–C_6H_5, *H4*), 8.10 and 8.30 (1H; s; –N=CH–). ESI-MS (*m*/*z*); 442 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}FN_5O_3$: C, 63.00; H, 4.33; N, 16.70. Found: C, 62.86; H, 4.66; N, 16.63.

*4.1.7.9. N′-(3-Fluorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**15**).* The derivative **15** was obtained as a white solid by condensation of **6** with 3-fluorobenzaldehyde in 90% yield, mp 255 °C. IR (KBr); 3640–3280 (N–H), 1698 (C=O, pyridazinone), 1673 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; $-CH_3$), 4.10 (2H; s; $-CH_2-C_6H_5$), 4.80 and 5.20 (2H; s; $-CH_2-$CONH–), 7.10–7.20 (6H; m; $-CH_2-C_6H_5$, –N=CH–C_6H_5, *H4*), 7.33–7.46 (3H; m; –N=CH–C_6H_5, *H2*, *H5*, *H6*), 7.89 and 8.08 (1H; s; –N=CH–), 11.79 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 442 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}FN_5O_3$: C, 63.00; H, 4.33; N, 16.70. Found: C, 62.93; H, 4.21; N, 16.61.

*4.1.7.10. N′-(4-Fluorobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**16**).* The derivative **16** was obtained as a white solid by condensation of **6** with 4-fluorobenzaldehyde in 89% yield, mp 252 °C. IR (KBr); 3620–3330 (N–H), 1700 (C=O, pyridazinone), 1670 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; $-CH_3$), 4.09 (2H; s; $-CH_2-C_6H_5$), 4.78 and 5.18 (2H; s; $-CH_2-$CONH), 7.10–7.18 (7H; m; $-CH_2-C_6H_5$, –N=CH–C_6H_5, *H3*, *H5*), 7.44–7.47 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 7.89 and 8.07 (1H; s; –N=CH–), 11.63 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 442 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{22}H_{18}FN_5O_3$: C, 63.00; H, 4.33; N, 16.70. Found: C, 62.96; H, 4.26; N, 16.63.

*4.1.7.11. N′-(2-Methylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**17**).* The derivative **17** was obtained as a white solid by condensation of **6** with 2-methylbenzaldehyde in 91% yield, mp 205 °C. IR (KBr); 3680–3270 (N–H), 1701 (C=O, pyridazinone), 1670 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.43 (3H; s; $-C_6H_4-CH_3$), 2.53 (3H; s; $-CH_3$), 4.23 (2H; s; $-CH_2-C_6H_5$), 4.92 and 5.31 (2H; s; $-CH_2-$CONH–), 7.22–7.31 (8H; m; $-CH_2-C_6H_5$, –N=CH–C_6H_5, *H3*, *H4*, *H5*), 7.76 (1H; dd; –N=CH–C_6H_5, *H6*, *J*: 8.4 Hz, *J*: 1.6 Hz), 8.29 and 8.47 (1H; s; –N=CH–). ESI-MS (*m*/*z*); 438 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{23}H_{21}N_5O_3$: C, 66.49; H, 5.09; N, 16.86. Found: C, 66.47; H, 5.15; N, 16.77.

*4.1.7.12. N′-(3-Methylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**18**).* The derivative **18** was obtained as a white solid by condensation of **6** with 3-methylbenzaldehyde in 94% yield, mp 227 °C. IR (KBr); 3600–3280 (N–H), 1698 (C=O, pyridazinone), 1671 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.33 (3H; s; $-C_6H_4-CH_3$), 2.53 (3H; s; $-CH_3$), 4.23 (2H; s; $-CH_2-C_6H_5$), 4.91 and 5.32 (2H; s; $-CH_2-$CONH–), 7.22–7.33 (7H; m; $-CH_2-C_6H_5$, –N=CH–C_6H_5, *H4*, *H5*), 7.47–7.53 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 7.99 and 8.17 (1H; s; –N=CH–). ESI-MS (*m*/*z*); 438 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{23}H_{21}N_5O_3$: C, 66.49; H, 5.09; N, 16.86. Found: C, 66.44; H, 4.88; N, 16.78.

*4.1.7.13. N′-(4-Methylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**19**).* The derivative **19** was obtained as a white solid by condensation of **6** with 4-methylbenzaldehyde in 96% yield, mp 230–1 °C. IR (KBr); 3680–3340 (N–H), 1703 (C=O, pyridazinone), 1672 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.19 (3H; s; $-C_6H_4-CH_3$), 2.40 (3H; s; $-CH_3$), 4.09 (2H; s; $-CH_2-C_6H_5$), 4.78 and 5.17 (2H; s; $-CH_2-$CONH–), 7.09–7.18 (7H; m; $-CH_2-C_6H_5$, –N=CH–C_6H_5, *H3*, *H5*), 7.44–7.47 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 7.86 and 8.03 (1H; s; –N=CH–). ESI-MS (*m*/*z*); 438 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{23}H_{21}N_5O_3$: C, 66.49; H, 5.09; N, 16.86. Found: C, 66.31; H, 5.10; N, 16.75.

*4.1.7.14. N′-(2-Trifluoromethylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**20**).* The derivative **20** was obtained as a white solid by condensation of **6** with 2-trifluoromethylbenzaldehyde in 87% yield, mp 129 °C. IR (KBr); 3600–3380 (N–H), 1669 (C=O, pyridazinone and hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.40 (3H; s; $-CH_3$), 4.10 (2H; s; $-CH_2-C_6H_5$), 4.82 and 5.23 (2H; s; $-CH_2-$CONH–), 7.09–7.18 (5H; m; $-CH_2-C_6H_5$), 7.47–7.51 (1H; m; –N=CH–C_6H_5, *H4*), 7.58–7.67 (2H; m; –N=CH–C_6H_5, *H3*, *H5*), 8.01 and 8.10 (1H; d; –N=CH–C_6H_5, *H6*, *J*: 7.6 Hz), 8.26 and 8.44 (1H; s; –N=CH–), 11.89 and 11.98 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 492 $[M + Na]^+$ (100%), 413, 254. Anal. Calcd. for $C_{23}H_{18}F_3N_5O_3$: C, 58.85; H, 3.86; N, 14.92. Found: C, 58.80; H, 3.89; N, 14.85.

*4.1.7.15. N′-(3-Trifluoromethylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**21**).* The derivative **21** was obtained as a white solid by condensation of **6** with 3-trifluoromethylbenzaldehyde in 92% yield, mp 229–30 °C. IR (KBr); 3620–3290 (N–H), 1700 (C=O, pyridazinone), 1673 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.51 (3H; s; $-CH_3$), 4.22 (2H; s; $-CH_2-C_6H_5$), 4.94 and 5.35 (2H; s; $-CH_2-$CONH–), 7.21–7.30 (5H; m; $-CH_2-C_6H_5$), 7.63–7.67 (1H; m; –N=CH–C_6H_5, *H5*), 7.75 (1H; d; –N=CH–C_6H_5, *H4*, *J*: 6.8 Hz), 8.00–8.05 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 8.10 and 8.29 (1H; s; –N=CH–), 11.95 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 492 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{23}H_{18}F_3N_5O_3$: C, 58.85; H, 3.86; N, 14.92. Found: C, 58.69; H, 3.65; N, 14.81.

*4.1.7.16. N′-(4-Trifluoromethylbenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**22**).* The derivative **22** was obtained as a white solid by condensation of **6** with 4-trifluoromethylbenzaldehyde in 81% yield, mp 219 °C. IR (KBr); 3670–3320 (N–H), 1703 (C=O, pyridazinone), 1670 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.54 (3H; s; $-CH_3$), 4.25 (2H; s; $-CH_2-C_6H_5$), 4.96 and 5.37 (2H; s; $-CH_2-$CONH–), 7.25–7.33 (5H; m; $-CH_2-C_6H_5$), 7.79–7.83 (2H; m; –N=CH–C_6H_5, *H3*, *H5*), 7.92–7.97 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 8.12 and 8.30 (1H; s; –N=CH–), 12.00 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 492 $[M + Na]^+$ (100%), 282, 254. Anal. Calcd. for $C_{23}H_{18}F_3N_5O_3$: C, 58.85; H, 3.86; N, 14.92. Found: C, 58.68; H, 3.86; N, 14.85.

*4.1.7.17. N′-(2-Methoxybenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**23**).* The derivative **23** was obtained as a white solid by condensation of **6** with 2-methoxybenzaldehyde in 87% yield, mp 210–1 °C. IR (KBr); 3640–3280 (N–H), 1695 (C=O, pyridazinone), 1680 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.52 (3H; s; $-CH_3$), 3.82 (3H; s; $-OCH_3$), 4.21 (2H; s; $-CH_2-C_6H_5$), 4.88 and 5.29 (2H; s; $-CH_2-$CONH–), 6.95–7.00 (1H; m; –N=CH–C_6H_5, *H5*), 7.08 (1H; d; –N=CH–C_6H_5, *H3*, *J*: 8.4 Hz), 7.21–7.32 (5H; m; $-CH_2-C_6H_5$), 7.36–7.41 (1H; m; –N=CH–C_6H_5, *H4*), 7.76 and 7.84 (1H; d; –N=CH–C_6H_5, *H6*, *J*: 7.6 Hz), 8.35 and 8.53 (1H; s; –N=CH–), 11.75 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 454 $[M + Na]^+$ (100%), 432 $[M + H]^+$, 282. Anal. Calcd. for $C_{23}H_{21}N_5O_4$: C, 64.03; H, 4.91; N, 16.23. Found: C, 64.01; H, 4.67; N, 16.15.

*4.1.7.18. N′-(3-Methoxybenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**24**).* The derivative **24** was obtained as a white solid by condensation of **6** with 3-methoxybenzaldehyde in 79% yield, mp 213 °C. IR (KBr); 3600–3310 (N–H), 1685 (C=O, pyridazinone and hydrazide). ^{1}H NMR

(DMSO-d_6, 400 MHz); δ 2.52 (3H; s; –CH_3), 3.77 (3H; s; –OCH_3), 4.21 (2H; s; –CH_2–C_6H_5), 4.91 and 5.31 (2H; s; –CH_2–CONH–), 6.94–6.99 (1H; m; –N=CH–C_6H_5, *H4*), 7.21–7.35 (8H; m; –CH_2–C_6H_5, –N=CH–C_6H_5, *H2*, *H5*, *H6*), 7.98 and 8.16 (1H; s; –N=CH–), 11.78 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 454 $[M + Na]^+$ (100%), 432 $[M + H]^+$, 282. Anal. Calcd. for $C_{23}H_{21}N_5O_4$: C, 64.03; H, 4.91; N, 16.23. Found: C, 63.86; H, 4.59; N, 16.25.

*4.1.7.19. N′-(4-Methoxybenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**25**).* The derivative **25** was obtained as a white solid by condensation of **6** with 4-methoxybenzaldehyde in 89% yield, mp 208 °C. IR (KBr); 3640–3360 (N–H), 1699 (C=O, pyridazinone). 1683 (C=O, hydrazide), ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.52 (3H; s; –CH_3), 3.77 (3H; s; –OCH_3), 4.21 (2H; s; –CH_2–C_6H_5), 4.89 and 5.28 (2H; s; –CH_2–CONH–), 6.96–6.99 (2H; m; –N=CH–C_6H_5, *H3*, *H5*), 7.21–7.30 (5H; m; –CH_2–C_6H_5), 7.60–7.64 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 7.95 and 8.13 (1H; s; –N=CH–). ESI-MS (*m*/*z*); 454 $[M + Na]^+$ (100%), 432 $[M + H]^+$, 282. Anal. Calcd. for $C_{23}H_{21}N_5O_4$: C, 64.03; H, 4.91; N, 16.23. Found: C, 63.70; H, 4.86; N, 16.17.

*4.1.7.20. N′-(2-Nitrobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**26**).* The derivative **26** was obtained as a white solid by condensation of **6** with 2-nitrobenzaldehyde in 83% yield, mp 186–7 °C. IR (KBr); 3640–3360 (N–H), 1683 (C=O, pyridazinone and hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.54 (3H; s; –CH_3), 4.24 (2H; s; –CH_2–C_6H_5), 4.95 and 5.31 (2H; s; –CH_2–CONH–), 7.23–7.31 (5H; m; –CH_2–C_6H_5), 7.66–7.80 (2H; m; –N=CH–C_6H_5, *H4*, *H5*), 8.04–8.13 (2H; m; –N=CH–C_6H_5, *H3*, *H6*), 8.40 and 8.63 (1H; s; –N=CH–), 12.04 and 12.09 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 469 $[M + Na]^+$ (100%), 297, 282. Anal. Calcd. for $C_{22}H_{18}N_6O_5$: C, 59.19; H, 4.06; N, 18.83. Found: C, 59.25; H, 3.96; N, 18.80.

*4.1.7.21. N′-(3-Nitrobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**27**).* The derivative **27** was obtained as a white solid by condensation of **6** with 3-nitrobenzaldehyde in 83% yield, mp 263 °C. IR (KBr); 3645–3360 (N–H), 1697 (C=O, pyridazinone), 1672 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.53 (3H; s; –CH_3), 4.24 (2H; s; –CH_2–C_6H_5), 4.96 and 5.37 (2H; s; –CH_2–CONH–), 7.23–7.32 (5H; m; –CH_2–C_6H_5), 7.70–7.74 (1H; m; –N=CH–C_6H_5, *H5*), 8.16 and 8.35 (1H; s; N=CH), 8.17–8.19 (1H; d; –N=CH–C_6H_5, *H6*, *J*: 8.0 Hz), 8.23–8.26 (1H; m; –N=CH–C_6H_5, *H4*), 8.52 (1H; s; –N=CH–C_6H_5, *H2*), 11.99 and 12.01 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 469 $[M + Na]^+$ (100%), 267. Anal. Calcd. for $C_{22}H_{18}N_6O_5$: C, 59.19; H, 4.06; N, 18.83. Found: C, 58.92; H, 3.83; N, 18.65.

*4.1.7.22. N′-(4-Nitrobenzylidene)-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5H)-yl)acetohydrazide (**28**).* The derivative **28** was obtained as a white solid by condensation of **6** with 4-nitrobenzaldehyde in 82% yield, mp 279 °C. IR (KBr); 3640–3320 (N–H), 1696 (C=O, pyridazinone), 1670 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.53 (3H; s; –CH_3), 4.23 (2H; s; –CH_2–C_6H_5), 4.96 and 5.37 (2H; s; –CH_2–CONH–), 7.23–7.31 (5H; m; –CH_2–C_6H_5), 7.98–8.00 (2H; m; –N=CH–C_6H_5, *H2*, *H6*), 8.25–8.30 (2H; m; –N=CH–C_6H_5, *H3*, *H5*), 8.14 and 8.32 (1H; s; N=CH), 12.06 (1H; s; –CO–NH–N=). ESI-MS (*m*/*z*); 469 $[M + Na]^+$ (100%). Anal. Calcd. for $C_{22}H_{18}N_6O_5$: C, 59.19; H, 4.06; N, 18.83. Found: C, 58.88; H, 4.09; N, 18.47.

4.2. Cyclooxygenase assay

The effect of the compounds on COX-1 (ovine) and COX-2 (human recombinant) enzymes was determined by measuring prostaglandin E_2 (PGE_2) using a COX Inhibitor Screening Kit (Catalog No. 560131) from Cayman Chemical, Ann Arbor, MI, USA.

Reaction mixtures were prepared in 100 mM Tris–HCl buffer, pH 8.0 containing 1 μM heme and COX-1 or COX-2 and preincubated for 10 min in a waterbath (37 °C). The reaction was initiated by the addition of 10 μM arachinodic acid (final concentration in reaction mixture 100 μM) After 2 min the reaction was terminated by adding 1 M HCl and PGE_2 was quantitated by an ELISA method. The test compounds were dissolved in DMSO and diluted to the desire concentration with 100 mM potassium phosphate buffer (pH 7.4). Following transfer to a 96-well plate coated with mouse anti-rabbit IgG, the tracer prostaglandin acetylcholine esterase and primary antibody (mouse anti PGE_2) were added. Plates were then incubated at room temperature overnight, reaction mixtures were removed and wells were washed with 10 mM potassium phosphate buffer containing 0.05% Tween 20. Ellman's reagent (200 μM) was added to each well and the plate was incubated at room temperature (exclusion of light) for 60 min, until the control wells yielded an OD = 0.3–0.8 at 412 nm. A standard curve with PGE_2 was generated from the same plate, which was used to quantify the PGE_2 levels produced in the presence of test samples. Results were expressed as a percentage relative to a control (solvent-treated samples).

4.3. Molecular modeling

Ligand preparation: The structures of eight different conformations of *N*′-benzylidene-2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-d]pyridazin-4(5*H*)-yl)acetohydrazide **7** were optimized using the hybrid density functional, B3LYP and the 6-31G(d) basis set as implemented in Gaussian 03 [22]. Harmonic frequency analysis at the same level of theory was carried out to verify that each conformation was a local minimum on the potential energy surface, and permitted the evaluation of zero point energies, entropies at 298 K and thermal corrections to the enthalpies. From these, the relative free energy of each conformer was calculated. The two most stable conformations are shown in Fig. 2. Two conformers of each ligand obtained from Gaussian calculations were used for docking study.

Docking: Docking studies were performed with MOE (The Molecular Operating Environment) Version 2009.10, software available from Chemical Computing Group Inc., 1010 Sherbrooke Street West, Suite 910, Montreal, Canada H3A2R7, http://www.chemcomp.com. The X-ray crystallographic structure of COX-2 complexed with 1-phenylsulfonamide-3-trifluoromethyl-5-(4-bromophenyl)pyrazole (SC-558) was obtained from the Protein Data Bank (PDB: 1CX2) [21]. Then properly protonated in the presence of its ligand using the Protonate 3D process in MOE. The default Triangle Matcher placement method, followed by molecular mechanics refinement and GBVI scoring, was used for the docking runs. 100 docking iterations were performed for two conformers of each ligand and the final refined poses were ranked by the MM/GBVI binding free energy estimation, written in the S field. The best score of each ligand–enzyme complexes was selected.

Acknowledgment

The authors gratefully acknowledge the financial support provided by Scientific Research Fund of Hacettepe University though Project 0501301001. One of the authors (AR) thanks the Natural Sciences and Engineering Council (NSERC) of Canada for financial support.

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