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Synthesis and in silico biological activity evaluation of new N-substituted pyrazolo-oxazin-2-one systems

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Abstract—Cyclisation of pyrazolo-β-enaminones 3 readily obtained from 4-aceto acetyl pyrazol 2 with triphosgene led to the formation of N-substituted pyrazolo-1,3-oxazin-2-ones 4 in good yields. Estimation of pharmacotherapeutic potential, possible molecule mechanisms of action, toxic/side effects and interaction with drug-metabolizing enzymes were made for synthesised compounds on the basis of prediction of activity spectra for substances (PASS) prediction results and their analysis by PharmaExpert software. COX inhibition predicted by PASS was confirmed by experimental evaluation.

1. Introduction

Oxazinones constitute an important class of heterocycles, which has attracted much synthetic interest due to their wide range of biological activities. Considerable attention has been focused on these compounds since the discovery of Efavirenz, a trifluoromethyl-1,3-oxazin-2-one which is a non-nucleoside reverse transcriptase inhibitor and a selective anti-HIV drug.[1] Several benzoxazinones exhibit diverse pharmacological properties, such as antagonism to progesterone receptor,[2] antitumour,[3] antiviral,[4] antithrombotic,[5] antimycobacterial,[6] anti-inflammatory,[7] antidiabetic and hypolipidaemic[8] effects. Further to these applications, they have also been reported as inhibitors of human leucocyte elastase[9] and serotonin reuptake.[10] Oxazinones have also been utilized as useful synthetic precursors for the preparation of some organic compounds.[11–13] On the other hand, it is well known that pyrazole derivatives are associated with various pharmaceutical activities.[14]

Our research group has been working for a long time on the development of new anti-inflammatory and antimicrobial agents. New thiazolidinone derivatives with antimicrobial activities have emerged as a result of this work.[15] Moreover, new thiazole Shiff bases, thiazole amides and thiazolidinones[16–18] have been found with good anti-inflammatory properties. In addition, we have successfully attempted to develop new nootropic agents.[19] More recently, our attempts have been focused on finding new anti-inflammatory compounds with combined activities. As inflammation is often a consequence of microbial infection, the production of agents with combined anti-microbial and anti-inflammatory action would be beneficial for the treatment of many diseases. Moreover, inflammation is thought to be involved in Alzheimer's disease and other degenerative disorders of CNS. The production of nootropics with combined anti-inflammatory activity would probably result in agents with improved properties.

As members of the oxazinones' family are known to exhibit a variety of properties including anti-inflammatory and antimicrobial action, we designed and synthesised a new series of oxazinone derivatives hoping to result in novel multifunctional molecules. The in silico biological activity evaluation of the compounds and the in vitro evaluation of the cyclooxygenase inhibitory action of

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the compounds as a first estimation of their anti-inflammatory potential are presented in this paper.

Novel pharmacological actions can be found for N-substituted pyrazolo-oxazin-2-ones on the basis of computer-aided drug discovery approach with computer program prediction of activity spectra for substances (PASS).[20–23] It is based on a robust analysis of structure–activity relationships[21] in a heterogeneous training set currently including about sixty thousand of biologically active compounds from different chemical series with about 4500 types of biological activity.

Since only the structural formula of chemical compound is necessary to obtain PASS predictions, this approach can be used at the earliest stage of investigation. There are many examples of successful use of PASS approach for finding new pharmacological agents.[24–29]

Thus, PASS application to pyrazolo-oxazin-2-ones was done in order to identify prospective pharmacological properties that could be confirmed by experimental studies. The analysis of PASS prediction results including estimation of potential pharmacotherapeutic effects, mechanisms of action, interaction with the main drug-metabolizing enzymes and side/toxic effects for new N-substituted pyrazolo-oxazin-2-ones is presented.

Consequently, the foregoing aspects have prompted us to synthesise new heterocyclic derivatives combining both pyrazol and 1,3-oxazinone in one molecular frame.

A variety of synthetic procedures leading to compounds with the oxazinone skeleton have been developed,[30] including solid-phase synthesis.[31]

Our research group reported earlier the synthesis of series of benzimidazoles, benzimidazolones and 1,5-benzodiazepines based on the use of enaminones resulting from 2-pyrone.[32] In continuation on these studies, we were interested in pyrazolo-β-enaminones **3** as precursors for the preparation of original pyrazolo-oxazinones.

2. Results and discussion

2.1. Synthesis

As an extension of our cyclisation strategy towards heterocyclic compounds, involving enaminone intermediates, we report in this work the synthesis of oxazin-2-ones bearing a pyrazole side chain in the 6 position, using as starting materials dehydroacetic acid which allowed access to different pyranobenzodiazepines.[33]

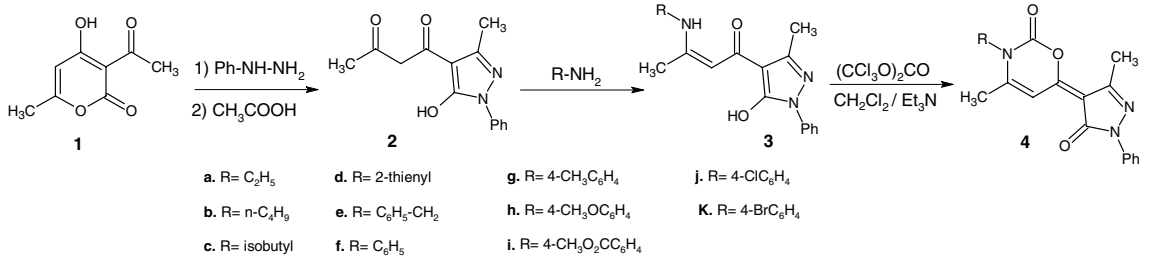
Scheme 1 outlines our general strategy developed to obtain the pyrazolo-oxazinones **4a–k**. This procedure starts with the reaction of the commercially available dehydroacetic acid **1** with phenylhydrazine to give in two steps the 1,3-dicarbonyl compound **2** according to the literature procedure.[34] Exposure of **2** to aliphatic and aromatic primary amines led to pyrazolo-enaminones **3a–k** using the method reported in the reference.[35] Ring closure of compounds **3a–k** with triphosgene in dichloromethane in the presence of triethylamine provided the target compounds **4a–k** in 70–85% yields (Table 1). The reaction mechanism could be rationalized as shown in Scheme 2.

All new structures of compounds **4a–k** were satisfactorily confirmed by ^{1}H and ^{13}C NMR mass spectrometry and elemental analysis. ^{13}C NMR spectra display in particular signals at around 158–161 and 164–166 ppm attributed, respectively, to the quaternary carbon of junction and carbonyl of pyrazolic nucleus. The main evidence for the formation of the oxazinone ring is the appearance of the carbamate carbonyl in the range of 152–156 ppm and the lack of carbonyl signal around 182–185 ppm. ^{1}H NMR spectrum indicates the absence of O–H signal which was in agreement with the proposed structures. The physico-chemical data of synthesised compounds are given in Table 1.

3. Biological activity spectra prediction

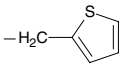
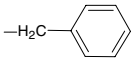
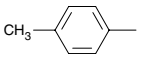
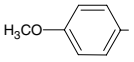
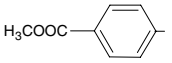
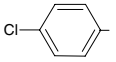
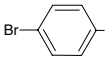
The analysis of biological activity spectra prediction for the synthesised compounds made in this publication is a good example of in silico study of chemical compounds before their experimental investigations. Anyone can do the same analysis using the free available web-site with the internet version of PASS and PharmaExpert: http://www.ibmc.msk.ru/PASS/.

A biological activity spectrum for a substance is a list of biological activity types for which the probability to be revealed (Pa) and the probability not to be revealed (Pi) are calculated. Pa and Pi values are independent and their values vary from 0 to 1. Biological activity spectra were predicted for all 11 synthesised structures with PASS 2005 version.[20] The result of prediction is valuable at planning of the experiment, but one should



Scheme 1. Synthesis of N-substituted 1,3-oxazin-2-one **4a–k**.

Table 1. Physicochemical data of N-substituted 1,3-oxazin-2-ones **4a–k** compounds

Compound	R	Yields (%)	Mp (°C)
4a	CH_3CH_2–	78	213
4b	$CH_3CH_2CH_2$–	76	217
4c	$(CH_3)_2CHCH_2$	74	210
4d		71	236
4e		72	224
4f		82	228
4g		85	220
4h		84	214
4i		70	216
4j		72	234
4k		72	230

take into account some additional factors: particular interest to some kinds of activity, desirable novelty of a substance, available facilities for experimental testing, etc. Actually, each choice is always the compromise between the desirable novelty of studied substance and risk to obtain the negative result in testing. The more is Pa value, the less is the probability of false positives in the set of compounds selected for biological testing. For example, if one selects for testing only compounds for which a particular activity is predicted with $Pa \geqslant 0.9$, the expected probability to find inactive compounds in the selected set is very low, but about 90% of active compounds are missed. If only compounds with $Pa \geqslant 0.8$ are chosen, the probability to find inactive compounds is also low, but about 80% of active compounds are missed etc. By default, in PASS Pa = Pi value is chosen as a threshold, therefore all compounds with Pa > Pi are suggested to be active. Another criterion for selection is the compounds' novelty. If Pa value is high, sometimes one may find close analogues of known biologically active substances among the tested compounds. For example, if Pa > 0.7 the chance to find the activity in experiment is high, but in some cases the compound may occur to be the close analogue of known pharmaceutical agents. If $0.5 < Pa < 0.7$ the chance to find the activity in experiment is less, but the compound is not so similar to known pharmaceutical agents. If $Pa < 0.5$ the chance to find the activity in experiment is even more less, but if it will be confirmed the compound might occur to be a New Chemical Entity.

PharmaExpert[36] was used for statistical and 'activity–activity' relationship analysis of PASS prediction results for the set of studied compounds. We calculated a number of activities predicted with different Pa values for the studied compounds (Fig. 1).

The figure shows that synthesised compounds may reveal greater number of biological activities (327 at Pa > 0.3; 98 at Pa > 0.5; 30 at Pa > 0.7). Keeping in mind how PASS results should be interpreted, we se-

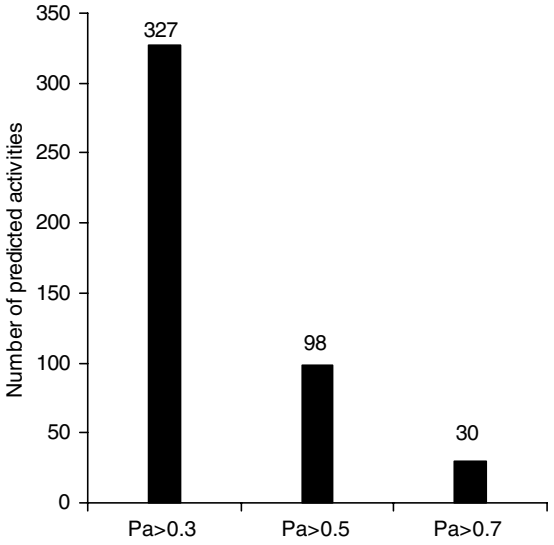
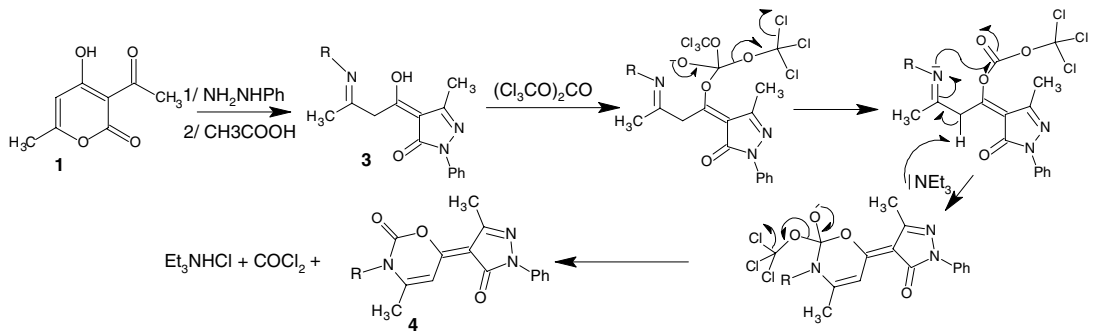


Figure 1. PASS prediction results for the studied compounds.



Scheme 2. Mechanism of synthesis.

lected predicted activities for studied compounds with Pa value more than 0.5. The result of selection obtained with computer program PharmaExpert is given in Table 2. Here, *Rank* reflects the position of predicted biological activity in the activity list arranged in descending order of biological activity occurrence for the analysed set of compounds; *N* is the number of compounds for which a particular biological activity is predicted.

As one may see from Table 2, the most frequently predicted types of biological activity are: amyotrophic lateral sclerosis treatment, analgesic, analgesic non-opioid, antiepileptic, anti-inflammatory, anxiolytic, cognition disorders treatment, corticotropin releasing factor antagonist, CYP2A1 substrate, CYP2A2 substrate, neuroprotector, nootropic, psychotropic, transplant rejection treatment, anticonvulsant, antipyretic, etc. All types of activity that are predicted for more than 2/3 of all compounds from the set can be considered as 'typical', all others—as 'minor'.[37] Since the purpose of this study was finding of new anti-inflammatory compounds, as well as COX inhibitors, one may conclude that anti-inflammatory (fifth rank) activity is typical. Such activities as antiasthmatic (rank 24) and others with higher rank are minor ones.

We also selected the types of activity that were predicted for a particular compound with the highest probability (focal activities). They are presented in Table 3.

Using PharmaExpert we analysed probable mechanisms of action for predicted 'typical' pharmacotherapeutical effects with Pa > 0.5 (Table 4). It may help to plan an experiment to find mechanism of predicted effects.

For example, 'amyotrophic lateral sclerosis treatment' activity was predicted with high probability for all compounds. At the same time the mechanism of amyotrophic lateral sclerosis treatment was predicted only for several compounds. Compounds **4a–c** were predicted as nerve growth factor agonists and compounds **4a–c**, **4e–g**, **4i**, **4j** were predicted as neurotrophic factors.

Analgestic and anti-inflammatory activity were predicted for all compounds with Pa > 0.5. Besides, all compounds were predicted as cyclooxygenase-1 inhibitors with Pa > 0.3. Five of them were predicted to be cyclooxygenase-1 inhibitors with Pa > 0.5 (**4f–h**, **4j**, **4k**).

We also predicted the toxic effects of the compounds. Only compound 4a has high probability to have many toxic/side effects with Pa > 0.5 (convulsant, eye/skin irritation, teratogenic and carcinogenic effect, acute toxicity, cardiotoxicity, hematotoxicity, embryotoxicity, arrhythmogenic effect, torsades de pointes). No other

Table 2. Analysis of biological activity predictions (Pa > 0.5) with Pharma Expert

Rank	*N*	Type of activity
1	11	Amyotrophic lateral sclerosis treatment
2	11	Analgesic
3	11	Analgesic, non-opioid
4	11	Antiepileptic
5	11	Anti-inflammatory
6	11	Anxiolytic
7	11	Cognition disorders treatment
8	11	Corticotropin releasing factor antagonist
9	11	CYP2A1 substrate
10	11	CYP2A2 substrate
11	11	Neuroprotector
12	11	Nootropic
13	11	Psychotropic
14	11	Transplant rejection treatment
15	10	Anticonvulsant
16	9	Antipyretic
17	9	Mediator release inhibitor
18	9	Tumour necrosis factor alpha antagonist
19	8	Antithrombocytopenic
20	8	Neurotrophic factor
21	6	Antiischaemic
22	5	Cyclooxygenase 1 inhibitor
23	4	Cyclooxygenase inhibitor
24	3	Antiasthmatic
...	...	...
70	1	Immunomodulator
...	...	...
98	1	Urologic disorders treatment

Table 3. Pa values for predicted biological activities

Compound	Focal predicted activity
4a	(−)-(4*S*)-Limonene synthase inhibitor (0.965)
4b	Nootropic (0.865)
4c	Nootropic (0.780)
4d	Amyotrophic lateral sclerosis treatment (0.799)
4e	Nootropic (0.849)
4f	Analgesic, non-opioid (0.843)
4g	Analgesic, non-opioid (0.829)
4h	Analgesic, non-opioid (0.804)
4i	Analgesic, non-opioid (0.771)
4j	Analgesic, non-opioid (0.828)
4k	Prolyl aminopeptidase inhibitor (0.879)

Table 4. 'Mechanism-effect' analysis of the prediction results with Pa > 0.5 for synthesised compounds

Effect	Mechanism of action
Amyotrophic lateral sclerosis treatment	Nerve growth factor agonist: **4a–c**[a] Neurotrophic factor: **4a–c**, **4e–g**, **4i**, **4j**
Nootropic	Nerve growth factor agonist: **4a–c**
Anti-inflammatory	Cyclooxygenase 1 inhibitor: **4f–h**, **4j**, **4k** Cyclooxygenase inhibitor: **4f–h**, **4j** Prostaglandin antagonist: **4j** Tumour necrosis factor alpha antagonist: **4a–c**, **4e–j**
Analgesic	Cyclooxygenase 1 inhibitor: **4f–h**, **4j**, **4k** Cyclooxygenase inhibitor: **4f–h**, **4j** Prostaglandin antagonist: **4j** Prostaglandin E1 antagonist: **4d** Tumour necrosis factor alpha antagonist: **4a–c**, **4e–j**
Neuroprotector	Nerve growth factor agonist: **4a–c** Nerve growth factor agonist: **4a–c** Neurotrophic factor: **4a–c**, **4e–g**, **4i**, **4j** Nootropic: **4a–k**
Anxiolytic	Corticotropin releasing factor antagonist: **4a–k**

[a] ID of compounds.

compound was predicted to have toxic/side effects at Pa > 0.5. It means that toxic/side effects of compound **4a** should be scrutinized in further investigation.

The PASS prediction results allow us to study the possible interactions with drug-metabolizing enzymes. It appeared that all synthesised compounds may be CYP2A1 and CYP2A2 substrates with high probability. 'CYP3A1 substrate' activity was predicted for compounds **4a**–**c**. Compound **4h** was predicted to be CYP2C9 inhibitor.

Thus, our study showed that prediction of biological activity spectra for synthesised compounds by PASS and its analysis made by PharmaExpert may estimate pharmacotherapeutic potential, possible molecular mechanisms of action, toxic/side effects and interaction with drug-metabolizing enzymes. These results may be used in further experimental studies.

4. Evaluation of cyclooxygenase inhibitory activity

As half of the compounds were predicted to inhibit cyclooxygenase-1 (COX-1) isoenzyme (Pa > 0.5), thus exhibiting anti-inflammatory and analgestic activity, we tested the inhibitory action of eight of the synthesised compounds (Table 5). Seven of the tested compounds exhibited COX-1 inhibitory activity when they were added at the reaction mixture at a concentration of 100 μM. Compound **4a** had no inhibitory effect when it was added at this concentration. However, it exhibited low inhibition (19.9%) at a final concentration of 200 μM. Compared to naproxen (inhibition: 39.3%), two of the tested compounds, **4f** and **4g**, showed higher inhibitory activity at the same experimental conditions. As gathered by the results, phenyl- (**4f**) and *p*-CH_3-phenyl groups are the substituents that mostly favor inhibitory action (inhibition: 61.8%, IC_{50} = 79.4 μM and 54.6%, IC_{50} = 91.2 μM, respectively). Ethyl- (**4a**) and *n*-propyl- (**4b**) substitution diminished inhibitory activity to 0% and 16%, respectively. *p*-OCH_3 (**4h**), *p*-Cl (**4j**) and *p*-Br (**4k**) substitution of the phenyl-ring lowered inhibition to 22.6%, 21.9% and 15%, as well. Phenyl-ring connection via a methylen-bridge (**4e**) also does not favor inhibition (inhibition: 18%). The four compounds exhibiting the best inhibitory action were among the five compounds predicted to be COX-1 inhibitors with Pa > 0.5.

Table 5. Experimental data and prediction results for the studied compounds

Compound	COX-1[a] inhibition %	IC_{50} (μM)	Pa
4a	0.0	—	—
4b	16.0	—	—
4e	18.0	—	—
4f	61.8	79.4	0.546
4g	54.6	91.2	0.541
4h	22.6	269	0.586
4j	21.9	251	0.563
4k	15.0	—	0.550
Naproxen	39.3		0.852

[a] Values are means of three determinations and deviation from the mean is <10% of the mean value.

Compound **4i** was not tested. Thus, PASS program in collaboration with PharmaExpert software successfully predicted COX-1 inhibition for the studied compounds.

In conclusion, we have developed a useful and efficient procedure for the preparation of new N-substituted pyrazolo-1,3-oxazin-2-ones as potent pharmaceutical agents. Furthermore, we have been able to use for the first time suitable pyrazolo-enaminones in cyclocondensation reaction with triphosgene. Studies directed towards the synthesis of thiazinones using this method are currently being pursued.

5. Experimental

All NMR spectra were recorded on a Brucker AC 2 300 spectrometer at 300 MHz (^1H) or 75 MHz (^{13}C), chemical shifts are expressed in δ (ppm) relative to TMS as internal standard. Mass data were acquired on a Nermag R10-10C mass spectrometer. Melting points were determined on a Buchi 512 Apparatus and was uncorrected. Microanalysis was performed at the INP-ENSIACET in Toulouse (France).

5.1. General procedure for the preparation of compounds 4

To a cooled (0 °C) solution of the appropriate enaminone **3** (5 mmol) and triethylamine (10 mmol) in 40 ml of dichloromethane, triphosgene (1.66 mmol) was slowly added dropwise under magnetic stirring. After being gradually warmed to room temperature, the resulting mixture was stirred for 48 h. Water was added, followed by extraction with dichloromethane. The organic layer was dried over sodium sulfate and evaporated to dryness to give the desired compounds **4a**–**k** as a white solid.

5.1.1. 3-Ethyl-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3,6-dihydro-2H-1,3-oxazin-2-one (4a). Yield: 78%; mp 213 °C; ^1H NMR (300 MHz, $CDCl_3$ + CF_3CO_2H) δ (ppm): 1.41 (t, 3H, J = 7.3 Hz), 2.57 (s, 3H), 2.65 (s, 3H), 3.56 (q, 2H), 5.30 (s, 1H), 7.28–7.97 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$ + CF_3CO_2H) δ (ppm): 14.0, 14.4, 18.3, 39.2, 96.5, 100.1, 120.8, 126.8, 129.3, 137.2, 145.1, 147.3, 154.0, 158.5, 165.0. MS (70 eV) *m*/*z* 311 (M^+, 26), 294 (2), 283 (1), 138 (20). Anal. Calcd for $C_{17}H_{17}N_3O_3$: C, 71.30; H, 5.46; N, 10.85. Found: C, 71.20; H, 5.39; N, 10.79.

5.1.2. 4-Methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-propyl-3,6-dihydro-2H-1,3-oxazin-2-one (4b). Yield: 76%; mp 217 °C; ^1H NMR (300 MHz, $CDCl_3$ + CF_3CO_2H) δ 1.08 (t, 3H, J = 7.3 Hz), 1.79 (sex, 2H, J = 7.3 Hz), 2.57 (s, 3H), 2.64 (s, 3H), 3.47 (m, 2H), 5.31 (s, 1H), 7.28–7.87 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$ + CF_3CO_2H) δ 11.5, 14.0, 18.4, 22.5, 46.1, 97.6, 100.5, 120.9, 126.8, 129.3, 137.2, 145.1, 147.3, 155.0, 159.0, 165.2. MS (70 eV) *m*/*z* 325 (M^+, 17), 310 (2), 283 (4), 152 (22). Anal. Calcd for $C_{18}H_{19}N_3O_3$: C, 71.80; H, 5.77; N, 10.47. Found: C, 71.77; H, 5.70; N, 10.40.

5.1.3. 3-Isobutyl-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3,6-dihydro-2H-1,3-oxazin-2-one (4c). Yield: 74%; mp 210 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 1.08 (d, 6H, $J = 6.9$ Hz), 2.04 (sex, 1H, $J = 6.9$ Hz), 2.56 (s, 3H), 2.64 (s, 3H), 3.33 (d, 2H, $J = 6.9$ Hz), 5.32 (s, 1H), 7.28–7.87 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 14.0, 18.5, 20.2, 28.6, 51.9, 97.2, 100.0, 120.8, 126.8, 129.3, 137.2, 145.2, 147.4, 155.5, 159.5, 165.0. MS (70 eV) *m/z* 339 (M^+, 31), 324 (7), 283 (35), 166 (6). Anal. Calcd for $C_{19}H_{21}N_3O_3$: C, 72.27; H, 6.06; N, 10.10. Found: C, 72.20; H, 6.00; N, 10.01.

5.1.4. 4-Methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-(2-thienylmethyl)-3,6-dihydro-2H-1,3-oxazin-2-one (4d). Yield: 70%; mp 236 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.59 (s, 3H), 2.70 (s, 3H), 5.01–5.08 (m, 2H), 5.32 (s, 1H), 6.93–7.54 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 13.74, 20.1, 44.8, 98.0, 104.0, 121.9, 127.4, 127.7, 129.9, 130.3, 138.9, 140.8, 145.4, 148.5, 156.0, 158.5, 166.0. MS (70 eV) *m/z* 379 (M^+, 2), 335 (1), 268 (1), 241 (2). Anal. Calcd for $C_{20}H_{17}N_3O_3S$: C, 63.31; H, 4.52; N, 11.07. Found: C, 63.27; H, 4.57; N, 11.01.

5.1.5. 3-Benzyl-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3,6-dihydro-2H-1,3-oxazin-2-one (4e). Yield: 72%; mp 224 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.61 (s, 3H), 2.71 (s, 3H), 4.85–4.93 (m, 2H), 5.31 (s, 1H), 7.27–7.73 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 13.80, 20.1, 44.7, 97.5.0, 105.0, 122.4, 128.3, 129.1, 129.2, 129.5, 130.0, 134.6, 144.2, 145.6, 149.3, 156.0, 158.4, 166.5. MS (70 eV) *m/z* 373 (M^+, 3), 329 (1), 267 (1), 241 (2). Anal. Calcd for $C_{22}H_{19}N_3O_3$: C, 70.67; H, 5.13; N, 11.25. Found: C, 70.72; H, 5.09; N, 11.21.

5.1.6. 4-Methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-phenyl-3,6-dihydro-2H-1,3-oxazin-2-one (4f). Yield: 80%; mp 228–229 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.05 (s, 3H), 2.56 (s, 3H), 5.31 (s, 1H), 7.23–7.77 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 16.70, 21.4, 98.1, 101.7, 121.5, 126.5 128.0, 129.0, 129.5, 129.8, 133.0, 137.9, 145.4, 149.1, 154.5, 159.2, 164.0. MS (70 eV) *m/z* 359 (M^+, 100), 314 (25), 313 (12), 200 (1). Anal. Calcd for $C_{21}H_{17}N_3O_3$: C, 70.18; H, 4.77; N, 11.69. Found: C, 70.10; H, 4.72; N, 11.64.

5.1.7. 4-Methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-(4-methylphenyl)-3,6-dihydro-2H-1,3-oxazin-2-one (4g). Yield: 85%; mp 220 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.09 (s, 3H), 2.54 (s, 3H), 2.61 (s, 3H), 5.39 (s, 1H), 7.14–7.99 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 17.00, 21.12, 21.20, 97.8, 102.7, 120.1, 125.4, 127.4, 128.9, 130.3, 132.7, 138.0, 140.7, 145.8, 148.2, 155.6 158.2, 164.6. MS (70 eV) *m/z* 373 (M^+, 58), 328 (14), 314 (2), 200 (15). Anal. Calcd for $C_{22}H_{19}N_3O_3$: C, 70.67; H, 5.13; N, 11.25. Found: C, 70.69; H, 5.09; N, 11.19.

5.1.8. 3-(4-Methoxyphenyl)-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3,6-dihydro-2H-1,3-oxazin-2-one (4h). Yield: 84%; mp 214 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 1.96 (s, 3H), 2.50 (s, 3H), 3.87 (s, 3H), 5.31 (s, 1H), 6.95–7.98 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 17.20, 21.20, 55.6, 97.7, 102.7, 115.3, 118.9, 124.4, 127.4, 138.7, 128.9.7, 138.8, 146.2, 147.5, 154.0 160.1, 160.5, 164.7. MS (70 eV) *m/z* 389 (M^+, 63), 313 (1), 344 (6), 200 (10). Anal. Calcd for $C_{22}H_{19}N_3O_4$: C, 67.86; H, 4.92; N, 10.79. Found: C, 67.76; H, 4.80; N, 10.71.

5.1.9. 4-[4-Methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-2-oxo-3,6-dihydro-2H-1,3-oxazin-3-yl]phenylacetate (4i). Yield: 70%; mp 216 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 1.91 (s, 3H), 2.50 (s, 3H), 3.98 (s, 3H), 5.30 (s, 1H), 7.14–8.24 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 17.20, 21.00, 52.60, 97.0, 103.2, 118.9, 124.5, 128.2, 128.7, 131.4, 131.9, 138.7, 139.2, 145.5, 147.4, 152.3 159.8, 164.5, 165.6. MS (70 eV) *m/z* 417 (M^+, 6), 372 (2), 314 (1), 200 (2). Anal. Calcd for $C_{23}H_{19}N_3O_5$: C, 66.18; H, 4.59; N, 10.07. Found: C, 66.12; H, 4.49; N, 10.01.

5.1.10. 3-(4-Chlorophenyl)-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-phenyl-3,6-dihydro-2H-1,3-oxazin-2-one (4j). Yield: 72%; mp 234 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.23 (s, 3H), 2.73 (s, 3H), 5.31 (s, 1H), 7.14–7.86 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 15.10, 22.3, 97.3, 100.04, 120.3, 122.1 123.6, 126.7, 128.5, 129.9, 132.1, 141.5, 146.2, 149.0, 156.0, 161.0, 165.0. MS (70 eV) *m/z* 393 (M^+, 48), 348 (14), 314 (5), 199 (18). Anal. Calcd for $C_{21}H_{16}N_3O_3Cl$: C, 64.05; H, 4.09; N, 10.67. Found: C, 64.03; H, 4.12; N, 10.70.

5.1.11. 3-(4-Bromophenyl)-4-methyl-6-(3-methyl-5-oxo-1-phenyl-1,5-dihydro-1H-4-pyrazolyliden)-3-phenyl-3,6-dihydro-2H-1,3-oxazin-2-one (4k). Yield: 72%; mp 230 °C; ^{1}H NMR (300 MHz, $CDCl_3 + CF_3CO_2H$) δ 2.28 (s, 3H), 2.74 (s, 3H), 5.31 (s, 1H), 7.09–7.67 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3 + CF_3CO_2H$) δ 15.40, 22.9, 97.2, 100.1, 122.4, 123.2, 124.7, 126.4, 128.8, 129.8, 133.5, 135.3, 146.2, 149.0, 155.7, 161.2, 166.0. MS (70 eV) *m/z* 439 (M^+, 26), 394 (5), 314 (7), 198 (25). Anal. Calcd for $C_{21}H_{16}N_3O_3Br$: C, 57.55; H, 3.68; N, 9.59. Found: C, 57.49; H, 3.59; N, 9.54.

5.2. Biological activity spectra prediction

Prediction of activity spectra for substances (PASS) is software for prediction of more than 2800 types of biological activity (PASS 2005 version) including pharmacotherapeutical effects, mechanisms of actions, interactions with drug-metabolizing enzymes, side and toxic effects on the basis of structural formula of chemical compounds. Average accuracy of prediction in leave-one-out cross-validation procedure (for ~68,000 compounds and ~2800 kinds of biological activity from the PASS training set) was about 93%. PharmaExpert is

software for analysis of PASS prediction results on the basis of activity–activity relationships. Details of the method used for biological activity prediction are presented at the web-site[17] and described in the publications.[21–24,28]

5.3. Experimental evaluation of biological activity

5.3.1. COX-1 inhibitor screening assay. The COX-1 activity of the compounds was measured using ovine COX-1 isoenzyme included in the 'COX Inhibitor Screening Assay' kit provided by Cayman (Cayman Chemical Co., Ann Arbor, MI, USA). The assay directly measures PGF_{2a} produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid production was quantified via enzyme immunoassay using a broadly specific antibody that binds to all the major prostaglandin compounds.[38] Arachidonic acid was added at the reaction mixture at a final concentration of 0.1 μM. The compounds were added at a final concentration of 100 μM unless otherwise mentioned. IC_{50} values were calculated for the most active compounds. Naproxen used as a positive control was added to the reaction mixture at the same concentration, 100 μM, as the tested compounds.

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