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Synthesis of Coumarin-Isoxazole-Pyridine Hybrids with Possible Biological Activity [1]

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Abstract: The 1,3 -dipolar cycloaddition reaction of nitrile oxides, prepared *in situ* from pyridine aldehyde oximes, with propargyloxy- or propargylaminocoumarins afforded the corresponding new 3,5-disubstituted isoxazoles in moderate to good yields. As oxidants for the formation of nitrile oxides utilized (diacetoxyiodo)benzene (PIDA) at room temperature or under microwave irradiation or tert-butyl nitrite (TBN) under reflux. Preliminary *in vitro* screening tests for some biological activities of the new compounds have been performed. Compounds **12b** and **13a** are potent LOX inhibitors with IC_{50} 5 μM and 10 μM, respectively, while hybrids **12b** and **13a** exhibit moderate to low anticancer activities on Hela, HT-29, and H1437 cancer cells.

Keywords: coumarin-isoxazole-pyridine hybrid; 1,3-dipolar cycloaddition reaction; propargyloxycoumarin; propargylaminocoumarin; pyridine aldehyde oxime; phenyliodine(III) diacetate (PIDA); *tert*-butyl nitrite (TBN); LOX inhibitors

1. Introduction

Coumarin derivatives with natural or synthetic origin represent a large variety of compounds with diverse biological and pharmacological properties [2-9]. These properties encompass anti-HIV [10], anticancer [11], antioxidant [12], anti-inflammatory [12,13], anti-Alzheimer [14], antidepressant [15], antibacterial [16], anticonvulsant [17], antitubercular [18], anticoagulant [19], etc. activities.

The hybrid drug concept is a sophisticated approach of combination therapy valuable in the treatment of complex and multifactorial diseases such as cancer, infectious and inflammatory diseases as well as neurological disorders, where traditional single-target therapy often is not satisfactory. Molecular hybridization is a rational drug design strategy that combines two or more pharmacophore groups into a single multi-functional molecule. The last decade, coumarin-isoxazole hybrids, among coumarin derivatives, have been synthesized, as they present various biological activities, such as antibacterial [20,21], anticancer [22,23], antiviral, anti-inflammatory, anti-psychotic, antidiabetic [23], antiproliferative [24], antimicrobial [25,26], anticoagulant and anticholinesterase [27]. Hybrids containing coumarin-pyridine scaffold exhibit, also, a plethora of biological activities, such as anticancer [28,29,30], anti-Alzheimer, antitubercular, antimicrobial, antiviral [31], anti-osteoporotic [32], and antileishmanial [33]. Additionally, isoxazole-pyridine hybrids present

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interesting biological properties, such as anti-acetylcholinesterase [34], anticancer, antioxidant [35], antitubercular [36], and inhibition of human cytochrome P-450 2A6 [37] activities.

An important method for the synthesis of isoxazole derivatives is the Huisgen 1,3-dipolar cycloaddition reaction of nitrile oxides to alkynes leading to the formation of 3,5-disubstituted isoxazoles [38,39]. The nitrile oxides are formed *in situ* from the corresponding aldoximes through chlorination and subsequent elimination of HCl by a base or oxidation of aldoxime using an oxidant [34,36]. (Diacetoxyiodo)benzene (PIDA) in room temperature (r.t.) [40] or under heating [36], or under microwave irradiation [41] is utilized for this oxidation. Other analogous reactions use hypochlorous acid at r. t. [34], or cerum (IV) ammonium nitrate (CAN) under sonication [36], or oxone at r.t. [36], or tert-butyl nitrite (TBN) under heating [36], or (bis(trifluoroacetoxy)iodo)benzene (PIFA) under heating [34] as oxidants.

As we can see from the above referred hybrids compounds containing together coumarin with isoxazole and pyridine moieties are unknown. According to our knowledge, small evidence is only given for coumarin hybrids with piperidine, dihydropyridine or tetrahydropyridine framework, Piperidine hybrids exhibit anti-filovirus [23,42], anti-psychotic [23,43], anti-acetylcholinesterase and anti-butyrycholinesterase [23,44] activities. 1,4-Dihydropyridine hybrids present antidiabetic activity [23,45]. 1,2,3,4-Tetrahydropyridine fused coumarin with isooxazoline hybrid has been synthesized by intramolecular 1,3-dipolar cycloaddition reaction [46]. In continuation of our ongoing interest in the synthesis and biological evaluation of coumarin hybrids [47-50], we would like to present herein the synthesis of some new coumarin-isoxazole-pyridine hybrids derived by 1,3-dipolar cycloaddition reaction of pyridine aldoximes with propargyloxy- or propargylaminocoumarins to study further their biological properties. The reactions studied and the isolated products are depicted in Schemes 1–2.

2. Results and Discussion

The 1,3-dipolar cycloaddition reaction of nitrile oxide, generated *in situ* from picolinaldehyde oxime (**2**) [51], with 1.1 equivalents of 7-propargyloxycoumarin (**1a**) [52] was selected as a model reaction for the investigation of suitable conditions (Scheme 1). At first, the reaction was performed using 1.1 equivalents of PIDA as the oxidant in methanol with 0.057 M concentration of oxime at room temperature (**Method A**) to give the new 3,5-disubstituted isoxazole derivative **3a** in 60% yield. The dimerization product, furoxan **4** [53] (20%) was also isolated from the reaction mixture (Table 1, entry 1). We tested, also, the above reaction with a 0.015 M concentration of oxime, but the results were quite similar. HSQC experiments revealed the regiochemistry of **3a**. The 4-H of isoxazole ring at 7.04 ppm corresponds to 103.1 ppm, as depicted from HSQC, both characteristic for 4-H and 4-C of 3,5-diaryl-substituted isoxazoles [54]. When microwave irradiation was used for this reaction in ethanol at 120°C for 1 h (**Method B**) isoxazole **3a** was obtained in 48% yield followed by furoxan **4** (16%) and 1,2,4-oxadiazole **5** [55] (9%) (Table 1, entry 2). Compound **5** was possibly synthesized by the 1,3-dipolar cycloaddition reaction of the nitrile oxide with the corresponding nitrile formed by dehydration of oxime **2** under the reaction conditions. As is known, aldehyde oximes can be converted to nitriles under microwave irradiation in the presence of alumina [56] or zeolite [57]. Then, we tested TBN as the oxidant for this reaction with acetonitrile as solvent under reflux for 18 h (**Method C**). Isoxazole **3a** (34%) and 1,2,4-oxadiazole **5** (32%) were isolated from the reaction mixture (Table 1, entry 3). It seems again that the increase in temperature favors the dehydration of oxime and the formation of 1,2,4-oxadiazole.

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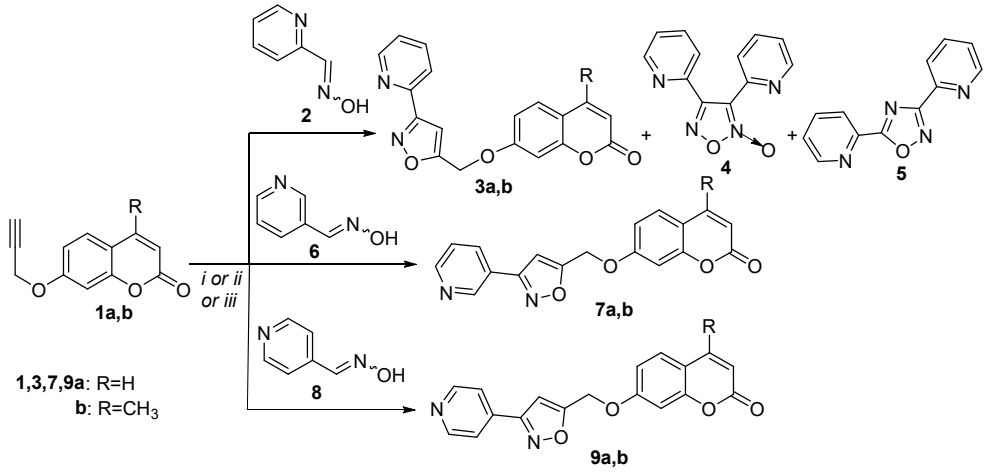

Scheme 1. Reaction conditions: *(i)* Method A: propargyl coumarin (1.1 equiv.), PIDA (1.1 equiv.), oxime (1 equiv.) 0.057 M in MeOH, r. t. or 0.015 M in MeOH and TFA (0.5 equiv.), 1 h – 2 d; *(ii)* Method B: propargyl coumarin (1.1 equiv.), PIDA (1.1 equiv.), oxime (1 equiv.), EtOH, MW, 120°C, 1 h or 100°C, 1 h or 2 h (for **11a** or **11b**); *(iii)* Method C: propargyl coumarin (1.1 equiv.), TBN (1.1 equiv.), oxime (1 equiv.), MeCN, 18 h.

Table 1. 1,3-Dipolar cycloaddition reactions of pyridine aldoximes with propargylcoumarins.

Entry	Oxime	Propagylcoumarin	Method[a]	Temperature	Time	Products (% yield)
1	**2**	**1a**	A	r. t.	1 h	**3a** (60), **4** (20)
2	**2**	**1a**	B	120°C	1 h	**3a** (48), **4** (16), **5** (9)
3	**2**	**1a**	C	Reflux	18 h	**3a** (34), **5** (32)
4	**2**	**1b**	A	r. t.	15 h	**3b** (65), **4** (17)
5	**2**	**1b**	B	120°C	1 h	**3b** (43), **4** (17), **5** (11)
6	**2**	**1b**	C	Reflux	18 h	**3b** (44), **4** (15), **5** (13)
7	**6**	**1a**	C	Reflux	18 h	**7a** (61)
8	**6**	**1b**	C	Reflux	18 h	**7b** (53)
9	**8**	**1a**	A[b]	Reflux	2 d	**9a** (24)
10	**8**	**1a**	C	Reflux	18 h	**9a** (42)
11	**8**	**1b**	C	Reflux	18 h	**9b** (45)
12	**2**	**10a**	B	100°C	1 h	**11a** (44), **4** (27)
13	**2**	**10a**	C	Reflux	18 h	**11a** (62), **4** (5), **5** (11)
14	**2**	**10b**	B	100°C	2 h	**11b** (55)
15	**6**	**10a**	A[b]	Reflux	2 d	**12a** (30)
16	**6**	**10a**	C	Reflux	18 h	**12a** (33)
17	**6**	**10b**	C	Reflux	18 h	**12b** (56)
18	**8**	**10a**	A[b]	Reflux	2 d	**13a** (55)
19	**8**	**10a**	C	Reflux	18 h	**13a** (40)

[a]: Methods A, B, C are referred in Scheme's 1 caption; [b]: TFA (50 mol%), ethanol.

The similar reactions of oxime **2** with 4-methyl-7-propargyloxycoumarin (**1b**) [52] under **Method A**, **B**, or **C** resulted in the synthesis and isolation of isoxazole **3b** in 65%, 43%, or 44% yield, respectively, along with furoxan **4** and 1,2,4-oxadiazole **5** (Table 1, entries 4-6). The next 1,3-dipolar cycloaddition reactions were of nitrile oxide, generated from nicotine aldehyde oxime (**6**) [37], with 7-propargylocoumarins **1a** and **1b** (Scheme 1)**.** Best results were under **Method C** and led to the synthesis of isoxazoles **7a** and **7b** in 61% and 53% yield, respectively (Table 1, entries 7,8). The yield of these reactions is in the common range of the reactions yield of the above nitrile oxide [37]. The reaction of nitrile oxide prepared from isonicotine aldehyde oxime (**8**) [58] in 0.015 M concentration with 7-propargyloxycoumarin (**1a**) under **Method A** did not give any product. When trifluoroacetic acid (TFA) 50% was added, the isoxazole **9a** was isolated, after 2 days, from the reaction mixture in

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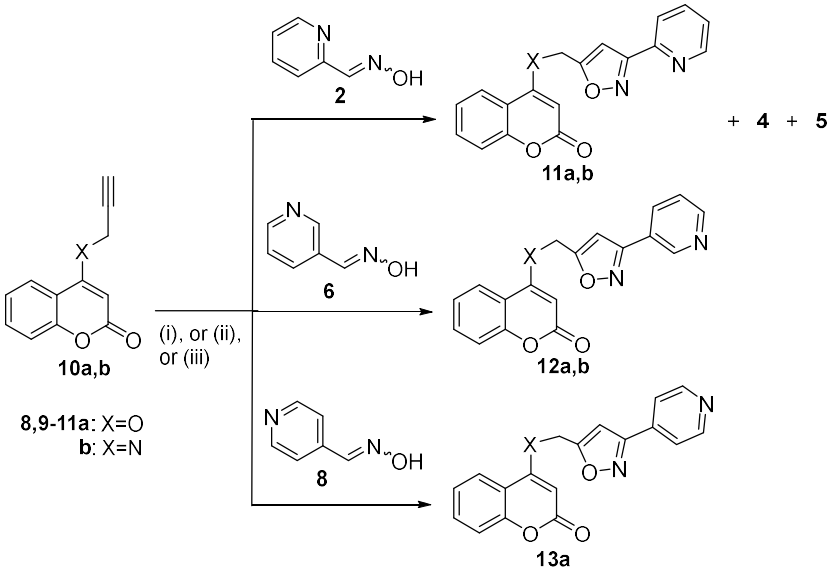
24% yield (Table 1, entry 9). This reaction under **Method C** led to isoxazole **9a** in 42% yield (Table 1, entry 10). The analogous reaction of oxime **8** with coumarin **1b** under **Method C** in r. t. did not give any results, while under reflux for 18 h afforded isoxazole **9b** in 45% yield (Table 1, entry 11).



Scheme 2. Reaction conditions: *(i), (ii), or (iii)* are referred in Scheme's 1 caption.

1,3-Dipolar cycloaddition reactions of 4-propargyloxycoumarin (**10a**) [59] and 4-propargylaminocoumarin (**10b**) [60] with the nitrile oxide, formed from pyridine aldoximes, were tested next (Scheme 2). The reaction of **10a** with **2** under **Method B** at 100°C afforded isoxazole **11a** (44%) and furoxan **4** (27%) (Table 1, entry 12), while under **Method C** resulted to **11a** in better yield (62%) accompanied by furoxan **4** (5%) and oxadiazole **5** (11%) (Table 1, entry 13). The similar reaction of **10b** with oxime **2** led to isoxazole **11b** (55%) under **Method B** (Table 1, entry 14). The reaction of oxime 6 with **10a** gave similar results under **Method A** with TFA or **Method C** resulted in isolation of isoxazole **12a** in 30% or 33% yield, respectively (Table 1, entries 15,16). Oxime **6** reacted, also, with 4-propargylaminocoumarin (**10b**) under **Method C** to give isoxazole **12b** in 56% yield (Table 1, entry 17). Oxime **8** was examined next for the reaction with **10a**. By using PIDA at room temperature (**Method A**) there were no results. The addition of TFA and after refluxing in ethanol for 2 days the isoxazole **13a** was isolated in 55% yield (Table 1, entry 18). The same reaction under **Method C** afforded isoxazole **13a** in 40% yield (Table 1, entry 19). The efforts for reaction of **10b** with oxime **8** under **Method A**, with PIDA and addition of TFA (50%), **Method B** or **Method C** were unsuccessful leaving unaffected the 4-propargylaminocoumarin (**10b**).

2.2. Biology

As we have mentioned above, isoxazole derivatives of coumarin or pyridine exhibit anticancer, antioxidant and anti-inflammatory activities [22,23,24,35]. Therefore, we examined preliminary synthesized derivatives for their biological activities. In this research, we evaluated *in vitro* a group of coumarin isoxazoles as inhibitors of lipid and as anti-inflammatories, as inhibitors of soybean lipoxygenase (sLOX).

Reactive oxygen species (ROS) as products from the cell metabolism are continiously produced in the human body. Some of them are characterized as highly toxic and various cellular enzymatic and non-enzymatic mechanisms offer a rapid detoxification. Their extreme reactivity and the tendency to induce chain reactions lead to pathological processes like inflammation, asthma, cardiovascular and neurological disorders. Lipid peroxidation is one of the major outcomes of ROS mediated injury. They directly damage membranes and generates a number of products that possess neurotoxic activity.

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We investigated the antioxidant power of the compounds as inhibitors of lipid peroxidation of linoleic acid sodium induced by a water-soluble 2,2-azobis(2-amidino-propane) hydrochloride that generate in vitro peroxyl radicals through spontaneous thermal decomposition. The derived experimental conditions resembled cellular lipid peroxidation due to the activity of the undertaken radicals. Trolox was used as a refe compound for comparative purposes (Table 2). Among the isoxazole coumarins derivative **12b** which is a 4-substituted amine, presents the highest activity (90.4%), whereas **9b, 11a, 9a, 12a, 11b and 3a,** follow with 86.6, 86, 83, 72 and 66% anti-lipid peroxidation ability. **13a** And **7a** exhibit lower activities while **3b** is actually inactive. Lipophilicity does not seem to influence activity

Lipoxygenase (LOX is the key enzyme in leukotriene biosynthesis. Leukotrienes, derived from the biotransformation of arachidonic acid catalyzed by 5-lipoxygenase (5-LOX). They are inflammatory mediators, causing inflammation, cancer, and stroke. LOXs play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer whereas cerebral ischemia-reperfusion triggers lipid peroxidation and inflammation. Inhibitors of LOX have attracted attention initially as potential agents for inflammatory diseases treatment and to certain types of cardiovascular diseases. Inhibition of LOX was performed by the UV absorbance based enzyme assay [61]. Perusal of the IC_{50} inhibition values in Table 2 shows two potent inhibitors **13a** and **7a**. Both are ethers from the 7- or 4-position of the coumarin ring and there are conjugated to a pyridyl group. The 4-pyridyl derivative **13a** is highly active (IC_{50} = 5 μM). No inhibition/low was shown by the other derivatives. The corresponding amino- substituted derivatives do not possess any activity. It seems that bulk and stereochemistry of the derivatives more than lipophilicity influence inhibition.

Table 2. *In vitro* Antioxidant Activity. Inhibition of soybean lipoxygenase.

Entry	Compounds[a]	Clog *P* [b]	LOX (%)/IC_{50} μM	ILPO (%)
1	**3a**	2.27	no	66
2	**3b**	2.77	no	0.6
3	**7a**	2.27	10 μM	42
4	**7b**	2.27	no	2
5	**9a**	2.77	38	83.6
6	**9b**	2.77	no	86.6
7	**11a**	2.27	no	86
8	**11b**	1.95	no	66
9	**12a**	2.01	10	72
10	**12b**	1.95	18	90.4
11	**13a**	2.01	5 μM	44.6
12	**NDGA**		0.45 μM	
13	**Trolox**			93

[a]Compounds tested at 100 μM. Values are means ± SD of three or four different determinations. Means within each column differ significantly ($p < 0.05$); no, no result was given under the reported experimental conditions; [b]Biobyte BioByte Corporation, C-QSAR database, 201 W Fourth Str., Suite # 204, Claremont CA 91711-4707, USA.

2.3. Biochemistry

Cytotoxic activity against three different cancer cell lines were examined for the more potent isoxazole derivatives **7a, 9a, 12b**, and **13a.** HeLa from cervical cancer, HT-29 from colon cancer, and H1437 from lang cancer were utilized to access the cytotoxic activity of those compounds by using the colorimetric method 3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) [62]. The results were expressed as EC_{50} (the concentration that causes 50% loss of cell viability) (Table 4). The results showed that **7a**, and **9a** exhibited EC_{50} values greater than 100 μM in all three cell lines, indicating low cytotoxicity (Table 3, entries 1,2). In contrast, **12b** demonstrated the highest cytotoxicity in HeLa cells (EC_{50} = 38.1 μM) and a similar effect in H1437 cells (EC_{50} = 47.3 μM), whereas it was less effective in HT-29 cells (EC_{50} = 96.5 μM) (Table 3, entry 3). Similarly, **13a** showed moderate

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cytotoxicity, with EC_{50} values of 44.2 μM, 65.8 μM, and 74.8 μM in HeLa, HT-29, and H1437 cells, respectively (Table 3, entry 4).

Table 4. Half maximal effective concentration (EC_{50} Values) of **7a, 9a, 12b, 13a** in HeLa, HT-29, and H1437 cancer cell lines. Results are presented a means of three independent experiments.

		EC_{50} (μM)		
Entry	**Compound**	**HeLa**	**HT-29**	**H1437**
1	**7a**	>100	>100	>100
2	**9a**	>100	>100	>100
3	**12b**	38.1	96.5	47.3
4	**13a**	44.2	65.8	74.8

3. Materials and Methods

3.1. Materials

All the chemicals were purchased from either Sigma-Aldrich Chemie GmbH (Eschenstr. 5, 82024 Taufkirchen, Germany) or Merck KGaA, (Frankfurter Strasse 250, 64293 Darmstadt, Germany). Melting points were determined with a Kofler hot stage apparatus and are uncorrected. IR spectra were obtained with a PerkinElmer Spectrum BX spectrophotometer as Nujol mulls. NMR spectra were recorded with an Agilent 500/54 (DD2) (500 MHz and 125 MHz for ^{1}H and ^{13}C, respectively) using TMS as an internal standard. J values are reported in Hz. Mass spectra were determined with an LCMS-2010 EV instrument (Shimadzu, Kyoto, Japan) under electrospray ionization (ESI) conditions. HRMS (ESI-MS) were recorded with a ThermoFisher Scientific (168 Third Avenue, Waltham, MA 02451, USA) model LTQ Orbitrap Discovery MS. Silica gel No. 60 (Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany) was used for column chromatography.

3.2. Chemistry

3.2.1. General Procedure of the 1,3-Dipolar Cycloaddition Reactions of Propargyl Coumarins with Pyridine Aldoximes. Synthesis of (3-(pyridin-2-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (3a)

Method A: 0.1 g (0.5 mmol) of 7-(prop-2-yn-1-yloxy)-2*H*-chromen-2-one (**1a**) was dissolved in methanol (4 mL) under stirring at room temperature. 0.161 g (0.5 mmol) of PIDA was then added. 56 mg (0.455) mmol of picolinaldehyde oxime (**2**) was dissoved in methanol (4 mL) and the solution was added dropwise at the solution of alkyne over a period of 2 hours. The reaction was monitored by TLC [hexane:ethyl acetate (EA) (3:1)]. The reaction was completed 1 hour after the addition of oxime. The crude mixture was evaporated and the residue was separated by column chromatography [hexane:EA (3:1) to EA] to afford 88 mg (60%) of **3a** and 22 mg (20%) of **4**.

Method B: 0.1 g (0.5 mmol) of **1a** was dissolved in ethanol (4 mL) under stirring at room temperature. 0.161 g (0.5 mmol) of PIDA and 56 mg (0.455 mmol) of **2** were added and the reaction mixture was irradiated under MW irradiation at 120°C for 1 hour. The reaction was monitored by TLC (3:1 hexane:EA). The mixture was filtered, the precipitate was washed with hexane (3x3 mL) and dried to give 70 mg (48%) of **3a**. The filtrate was evaporated and purified by column chromatography [hexane:EA (3:1) to EA] to give 18 mg (16%) of **4** and 10 mg (9%) of **5**.

Method C: In a solution of 0.1 g (0.5 mmol) of **1a** in acetonitrile (8 mL), 56 mg (0.455 mmol) **2**) and 0.006 mL (52 mg, 0.5 mmol) of TBN were added under N_2 atmosphere at room temperature. The reaction was monitored by TLC [hexane:EA (3:1)]. The reaction was refluxed for 20 hours. After the completion of the reaction, as indicated by TLC, water (10 mL) was added. The precipitate formed was purified by Column Chromatography [hexane:EA (1:1) to EA] to give 50 mg (34%) of **3a**. The filtrate was extracted with EA (3x15 mL) and the combined organic layers was washed once with brine (20 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The crude mixture was purified by column chromatography [hexane:EA (3:1)to EA] to afford 33 mg (32%) of **5**.

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3a, White solid, m.p. 178-179°C (Hexane/Ethyl Acetate). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 5.30 (s, 2H), 6.29 (d, *J* = 9.5 Hz, 1H) 6.93 (s, 1H), 6.93 (d, *J* = 8.1 Hz, 1H), 7.04 (s, 1H), 7.33 – 7.38 (m, 1H), 7.42 (d, *J* = 8.2 Hz, 1H), 7.65 (d, *J* = 9.5 Hz, 1H), 7.81 (t, *J* = 7.2 Hz, 1H), 8.09 (d, *J* = 7.8 Hz, 1H), 8.68 (d, *J* = 4.2 Hz, 1H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ: 61.6, 102.2, 103.1, 112.9, 113.6, 114.1, 121.9, 124.9, 129.2, 137.1, 143.3, 148.1, 149.9, 155.8, 160.8, 161.0, 163.6, 167.4. IR (Nujol): 3086, 1728, 1629 cm^{-1}. LC-MS (ESI): (m/z): 343 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4Na$: 321.0870 $(M+Na)^+$; found: 321.0898.

4-Methyl-7-((3-(pyridin-4-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**3b**). Mass of 99 mg (65% under Method A), 65 mg (43% under Method B), 67 mg (44% under Method C), white solid, m.p. 173-175°C, (hexane/ethyl acetate). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.41 (s, 3H), 5.31 (s, 2H), 6.17 (s, 1H), 6.83 – 7.0 (m, 2H), 7.29 (s, 1H), 7.45– 7.53 (m, 1H), 7.55 (d, *J* = 8.7 Hz, 1H), 7.95 (t, *J* = 7.0 Hz, 1H), 8.20 (d, *J* = 7.9 Hz, 1H), 8.73 (d, *J* = 4.4 Hz, 1H). ^{13}C-NMR (126 MHz, DMSO-d_6) δ: 18.2, 60.6, 101.8, 103.4, 11.7, 112.5, 113.9, 121.4, 125.3, 126.7, 137.6, 147.3, 150.0, 153.4, 154.6, 160.0, 160.3, 162.9, 168.1. IR (Nujol): 3085, 1725, 1621 cm^{-1}. LCMS (ESI): (m/z): 357 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{19}H_{14}N_2O_4H$: 335.1026 $(M+H)^+$; found: 335.1054.

7-((3-(Pyridin-3-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**7a**). Mass of 89 mg (61 % under Method C), white solid, m.p. 169-171°C (hexane/ethyl acetate). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 5.51 (s, 2H), 6.34 (d, *J* = 9.5 Hz, 1H), 7.09 (dd, *J* = 8.6, 2.5 Hz, 1H), 7.20 (d, *J* = 2.4 Hz, 1H), 7.37 (s, 1H), 7.58 (dd, *J* = 8.0, 4.8 Hz, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 8.02 (d, *J* = 9.5 Hz, 1H), 8.30 (d, *J* = 7.9 Hz, 1H), 8.71 (dd, *J* = 4.8, 1.5 Hz, 1H), 9.10 (d, *J* = 1.6 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 60.9, 101.8, 102.8, 109.6, 112.9, 113.1,124.3, 124.4, 129.7, 134.4, 144.3, 147.5, 151.2, 155.2, 159.9, 160.2, 160.5, 168.3. IR (Nujol): 3084, 1710, 1625 cm^{-1}. LCMS (ESI): (m/z): 321$[M+H]^+$, 343 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4H$: 321.0870 $(M+H)^+$; found: 321.0866.

4-Methyl-7-((3-(pyridin-3-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**7b**). Mass of 81 mg (53 % under Method C), white solid, m.p. 150-152°C, (hexanee/ethylacetate). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.40 (s, 3H), 5.29 (s, 2H), 6.17 (s, 1H), 6.75 (s, 1H), 6.92 (d, *J*=2.0 Hz 1H), 6.95 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.42 (dd, *J* = 7.4, 5.0 Hz, 1H), 7.55 (d, *J* = 8.7 Hz, 1H), 8.16 (d, *J* = 7.8 Hz, 1H), 8.70 (d, *J* = 3.3 Hz, 1H), 9.02 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 18.8, 61.5, 101.8, 102.1, 112.5, 112.9, 114.8, 124.0, 124.9, 126.1, 134.3, 148.0, 151.3, 152.4, 155.2, 160.2, 160.5, 161.1, 168.1. IR (Nujol): 3090, 1710, 1620 cm^{-1}. LCMS (ESI): (m/z): 357 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{19}H_{14}N_2O_4H$: 335.1026 $(M+H)^+$; found: 335.1018.

7-((3-(Pyridin-4-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**9a**). Mass of 35 mg (24 % under Method A, with TFA), 61 mg (42% under Method C), white solid, m.p. 181-183°C, (hexane/ethyl acetate). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 5.38 (s, 2H), 6.20 (d, *J* = 9.5 Hz, 1H), 6.95 (dd, *J* = 8.6, 2.4 Hz, 1H), 7.00 (d, *J* = 2.4 Hz, 1H), 7.18 (s, 1H), 7.50(dd, *J* = 8.6, 3.5 Hz, 1H), 7.78 (dd, *J* = 9.2, 4.6 Hz, 1H), 7.96 (d, *J* = 4.1 Hz, 2H), 8.77 (d, *J* = 4.1 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$/DMSO-d_6) δ: 60.6, 101.4, 102.4, 106.3, 112.3, 112.88, 112.94, 121.6, 129.0, 143.3, 147.8, 155.1, 159.5, 159.9, 160.1. IR (Nujol): 3090, 1715, 1628 cm^{-1}. LCMS (ESI): (m/z): 321$[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4H$: 321.0870 $(M+H)^+$; found: 321.0857. m/z calcd. for $C_{18}H_{12}N_2O_4Na$: 343.0689 $(M+Na)^+$; found: 343.0684.

4-Methyl-7-((3-(pyridin-4-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**9b**). Mass of 68 mg (45% under Method C), white solid, m.p. 180-181°C, (hexane/ethyl acetate). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 2.41 (s, 3H), 5.53 (s, 2H), 6.25 (s, 1H), 7.10 (d, *J* = 8.7 Hz, 1H), 7.18 (s, 1H), 7.38 (s, 1H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.87 (d, *J* = 5.1 Hz, 2H), 8.75 (d, *J* = 5.0 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 18.1, 60.8, 101.8, 102.9, 111.7, 112.5, 113.9, 120.9, 126.7, 128.2, 128.9, 135.4, 150.7, 153.3, 154.6, 160.0, 160.3, 160.5, 168.8. IR (Nujol): 3086, 1718, 1630 cm^{-1}. LCMS (ESI): (m/z): 357 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{19}H_{14}N_2O_4H$: 335.1026 $(M+H)^+$; found: 335.1015.

4-((3-(Pyridin-2-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**11a**). Mass of 64 mg (44% under Method A), 90 mg (62% under Method C), white solid, m.p. 164- 165°C, (hexane/ethyl acetate). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 5.67 (s, 2H), 6.20 (s, 1H), 7.34 (s, 1H), 7.38 (t, *J* = 7.6 Hz, 1H), 7.43 (d, *J* = 8.3 Hz, 1H), 7.54 – 7.59 (m, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.86 (d, *J* = 7.9 Hz, 1H), 7.99 (td, *J* = 7.7, 1.1 Hz, 1H), 8.07 (d, *J* = 7.8 Hz, 1H), 8.74 (d, *J* = 4.2 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 61.6, 91.7, 103.7, 114.8, 116.5, 121.5, 122.9, 124.4, 125.3, 133.0, 137.6, 147.2, 150.0, 152.8, 161.4, 163.0, 164.0, 166.9. IR

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(Nujol): 3070, 1725, 1620 cm^{-1}. LCMS (ESI): (m/z): 343 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4H$: 321.0870 $(M+H)^+$; found: 321.0883.

4-(((3-(Pyridin-2-yl)isoxazol-5-yl)methyl)amino)-2H-chromen-2-one (**11b**).Mass of 80 mg (55% under Method B), whitish solid, m.p. 196-198°C, (hexane/ethyl acetate). ^1H NMR (500 MHz, $CDCl_3$) δ: 5.38 (s, 2H), 5.83 (s, 1H), 7.13 (s, 1H), 7.28 (t, *J* = 7.6 Hz, 1H), 7.33 (d, *J* = 8.2 Hz, 1H), 7.38 (dd, *J* = 6.9, 5.3 Hz, 1H), 7.57 (d, *J* = 7.7 Hz, 1H), 7.72 – 7.90 (m, 1H), 8.11 (d, *J* = 7.8 Hz, 1H), 8.69 (d, *J* = 4.4 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 61.7, 91.6, 103.7, 115.3, 117.0, 121.9, 123.2, 124.3, 125.0, 133.0, 137.2, 147.9, 149.9, 153.5, 162.4, 163.7, 164.7, 165.7; IR (Nujol): 3288, 3085, 1711, 1629 cm^{-1}. LCMS (ESI): (m/z): 343 $[M+H+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{13}N_3O_3H$: 320,103 $(M+H)^+$; found: 320.1057.

4-((3-(Pyridin-3-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**12a**). Mass of 44 mg (30% under Method A, TFA), 48 mg (33% under Method C),white solid, m.p. 189-191°C, (hexane/ethyl acetate). ^1H NMR (500 MHz, $CDCl_3$) δ: 5.38 (s, 2H), 5.84 (s, 1H), 6.85 (s, 1H), 7.31 (t, *J* = 7.5 Hz, 1H), 7.36 (d, *J* = 8.3 Hz, 1H), 7.46 (dd, *J* = 6.9, 4.8 Hz, 1H), 7.59 (t, *J* = 7.6 Hz, 1H), 7.86 (d, *J* = 7.9 Hz, 1H), 8.20 (d, *J* = 7.7 Hz, 1H), 8.73 (d, *J* = 3.3 Hz, 1H), 9.05 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$/ DMSO-d_6) δ: 61.2, 91.3, 102.7,114.7, 116.0, 123.7, 127.7, 128.5, 132.3, 145.4, 148.6, 152.6, 157.2, 159.0, 161.3, 163.8, 166.5. IR (Nujol): 3080, 1712, 1630 cm^{-1}. LCMS (ESI): (m/z): 343 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4H$: 321.0870 $(M+H)^+$; found: 321.0893.

4-(((3-(Pyridin-3-yl)isoxazol-5-yl)methyl)amino)-2H-chromen-2-one (**12b**). Mass of 9 mg (6% under Method A, TFA), 32 mg (22% under Method C), white solid, m.p. 197-199°C, (hexane/ethyl acetate); ^1H NMR (500 MHz, DMSO-d_6) δ: 4.79 (s, 2H), 5.31 (s, 1H), 7.17 (s, 1 H), 7.34 (d, *J*=8.4 Hz, 1H), 7.37 (t, *J* = 7.3 Hz, 1H), 7.53 (dd, *J* = 7.9, 4.8 Hz, 1H), 7.63 (t, *J* = 8.3 Hz, 1H), 8.10 (d, *J* = 8.0 Hz, 1H), 8.2 – 8.30 (m, 1H), 8.45 (s, 1H), 8.68 (d, *J* = 3.5 Hz, 1H), 9.07 (d, *J* = 1.3 Hz, 1H), ^{13}C NMR (126 MHz, DMSO-d_6) δ: 38.1, 83.1, 100.9, 114.4, 117.0, 122.6, 123.6, 124.2, 124.5, 132.2, 134.1, 147.5, 151.2, 153.09, 153.12, 159.8, 161.4, 170.2; IR (Nujol): 3320, 3085, 1710, 1625. cm^{-1}. LCMS (ESI): (m/z): 342 $[M+Na]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{13}N_3O_3H$: 320.1030 $(M+H)^+$; found: 320.1030.

4-((3-(Pyridin-4-yl)isoxazol-5-yl)methoxy)-2H-chromen-2-one (**13a**). Mass of 80 mg (55% under Method A), 58 mg (40% under Method C), m.p. 167-169°C, (hexane/ethyl acetate). ^1H NMR (500 MHz, DMSO-d_6) δ: 5.67 (s, 1H), 6.17 (s, 1H), 7.39 (t, *J* = 7.6 Hz, 1H), 7.44 (d, *J* = 8.3 Hz, 1H), 7.52 (s, 1H), 7.68 (d, *J* = 7.7 Hz, 1H), 7.83 – 7.92 (m, 1H), 8.77 (d, *J* = 4.8 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ: 61.9, 91.8, 103.1, 114.8, 116.5, 121.0, 123.0, 124.4, 133.0, 135.3, 150.7, 152.8, 160.7, 161.4, 163.9, 167.7. IR (Nujol): 3090, 1704, 1640 cm^{-1}. LCMS (ESI): (m/z): 321 $[M+H]^+$. HRMS (ESI): m/z calcd. for $C_{18}H_{12}N_2O_4H$: 321.0870 $(M+H)^+$; found: 321.0871.

3.3. Biological Experiments

The in vitro assays were performed at a concentration of 100 μM (a 10 mM stock solution in DMSO was used, from which several dilutions were made for the determination of IC_{50} values), at least in triplicate, and the standard deviation of absorbance was less than 10% of the mean. The compounds were diluted in 0.1% DMSO under sonification in an appropriate buffer in several dilutions (Table 2). Statistical comparisons were made using the Student T-test. A statistically significant difference was defined as $p < 0.05$.

3.3.1. Inhibition of Linoleic Acid Peroxidation

The *in vitro* study was evaluated as reported previously by our group [40]. 10 microliters of the 16 mM sodium linoleate solution were added to the UV cuvette containing 0.93 mL of a 0.05 M phosphate buffer, pH 7.4, pre-thermostated at 37°C. The oxidation reaction was initiated at 37°C under air by the addition of 50 μL of a 40mMAAPH solution, which was used as a free-radical initiator. Oxidation was carried out in the presence of the samples (10 μL from the stock solution of each compound) in the assay without antioxidants and monitored at 234 nm. Lipid oxidation was recorded in the presence of the same level of DMSO and served as a negative control. Trolox was used as the appropriate reference compound (Table 2).

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3.3.2. Soybean Lipoxygenase Inhibition Study

The *in vitro* study was evaluated as reported previously by our group [59]. The tested compounds were incubated in a tris buffer pH 9, at room temperature, with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution (1/9 x 10^{-4} w/v in saline, 1000 U/mL) for 5 min, and after that the inhibition was measured. The method was based on the conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm by the appearance of the conjugated diene. Nor-dihydroguaeretic acid NDGA (IC_{50} = 0.45 μM) was used as a reference compound. Different concentrations were used to determine the IC_{50} values. A blank determination was used first to serve as a negative control. The results are given in Table 2.

3.4. Biochemical Experiments

3.4.1. Cell Culture

HeLa (cervical cancer), HT-29 (colorectal cancer), and H1437 (lang adenocarcinoma) cell Lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimytotic and cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

3.4.2. Cytotoxicity Evaluation

Cell viability was assessed using the MTT assay. Briefly, cells were seeded in 96-well plates at a density of 3x10^3 cells per well for HeLa and H1437, and 4x10^3 cells per well for HT-29, in 100 μL of complete medium. After 24 hours of incubation for cell attachment, the cells were treated with varying concentrations of test compounds (ranging fro 10 to 100 μM) for 48 hours. Following the treatment period, MTT colorimetric assay was performed as described before [62]. Cell viability was calculated as a percentage of untreated control. The half-maximal inhibitory concentration (EC_{50}) values defined as the concentration that causes 50% reduction in cell viability relative to controls. All experimentes were performed in triplicate and repeated indepentedly at least three times.

5. Conclusions

Coumarin-isoxazole hybrids connected to pyridine framework have been synthesized in moderate to good yield by the 1,3-dipolar cycloaddition reaction of nitrile oxides, derived *in situ* from pyridine aldehyde oximes under oxidation by PIDA or TBN, with 4- or 7-propargyloxycoumarins or 4-propargylaminocoumarins. The 4-pyridyl hybrid **13a** is a potent inhibitor of LOX with IC_{50} = 5 μM, followed by the 3-pyridyl hybrid **7a** with IC_{50} = 10 μM. . Since Lipoxygenases and their catalysis products are related with carcinogenic process such as cell proliferation, differentiation and apoptosis the potent hybrid **13a,** will be used as a lead compound for further theoretical structural modifications and *in vitro* assays. Compounds **12b** and **13a** presented moderate to minimal impact on HeLa, HT-29, and H1437 cancer cells. Hybrid **12b** presents a combination of high antilipid peroxidation and a moderate impact on HeLa cells and will lead to the design of new hybrids with higher inpact on HeLa. Further investigation is in progress to delineate the role of these hybrids on inflammation.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org. ^{1}H-NMR and ^{13}C-NMR spectra of the compounds.

Author Contributions: Conceptualization, writing—original draft preparation, supervision, K.E.L.; performed the biological tests, review and editing the manuscript, D.J.H.-L.; performed the experiments, writing, M. D. D.; performed the biochemical tests, review and editing the manuscript, I. M. S.; review the manuscript and editing the manuscript, E. N.; performed the HRMS, C. G.

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Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DMSO	Dimethyl Sulfoxide
SD	Standard Deviation
TLC	Thin Layer Chromatography

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