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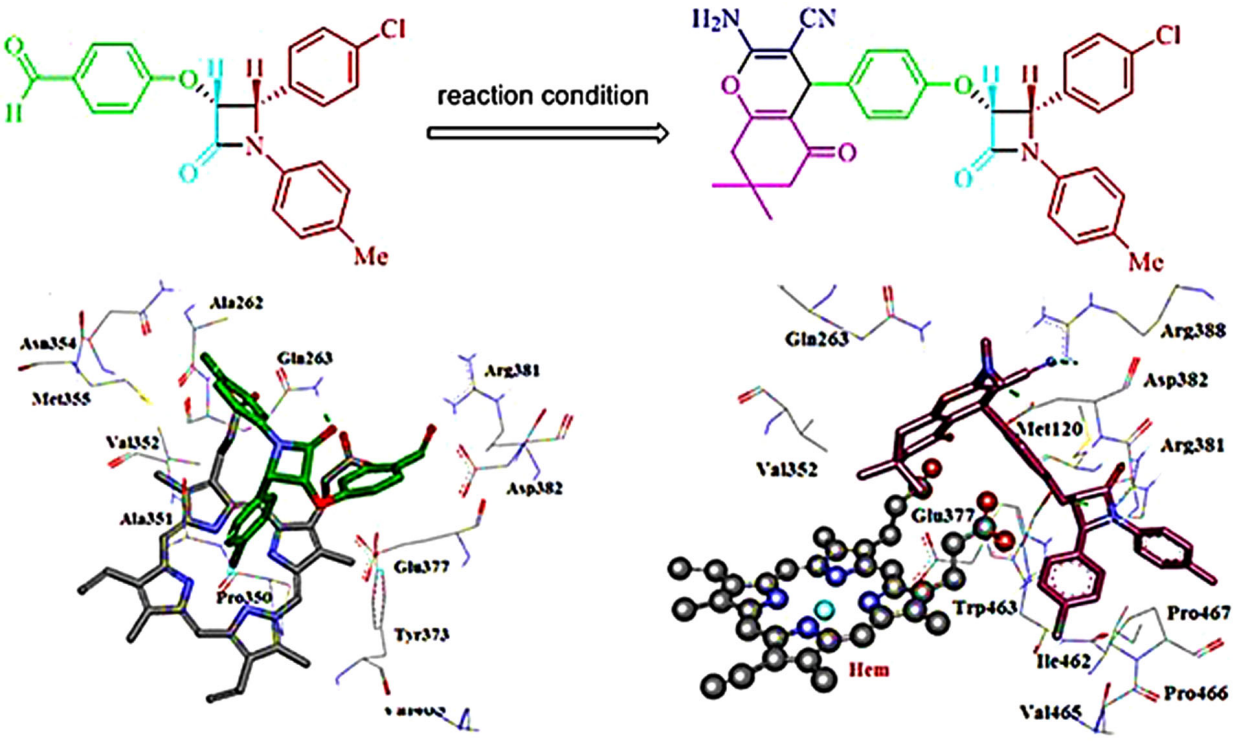

Binding modes of selected ligands with human inducible nitric oxide synthase active site

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Three-Component Synthesis of Chromeno β-Lactam Hybrids for Inflammation and Cancer Screening

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Abstract: Highly diastereoselective synthesis of chromeno β-lactam hybrids was achieved by an efficient one-pot three-component reaction. With this procedure, the desired β-lactam products were obtained in good yields and with exclusive *cis* stereoselection, by combining a variety of benzaldehydes, malononitrile, and either 5,5-dimethylcyclohexane-1,3-dione or 4-hydroxycoumarin in the presence of 1,4-diazabicyclo[2.2.2]octane under reflux conditions. These adducts were structurally characterized on the basis of IR, 1D and 2D NMR spectra, X-ray analysis, H-H COSY and H-C HSQC two-dimensional NMR experiments, and elemental analysis. Each of the synthesized compounds was screened for anti-inflammatory and anticancer activities. β-Lactams **5b** and **8b** showed a 53.4 and 19.8 anti-inflammatory ratio, respectively, and **5b** appeared more active than the well-known dexamethasone corticosteroid used for the treatment of rheumatoid and skin inflammation. β-Lactams **5a**, **5b**, **5e**, **5f**, **5g**, **8c**, **8j** and **8p** also showed good antitumor activity against the *SW1116* (colon cancer) cell line without notable cytotoxicity towards the *HepG2* control cell line.

Keywords: Anticancer, Anti-inflammatory, β-Lactams, Colon cancer, Cytotoxicity, DABCO.

1. Introduction

In the recent past, our laboratories have pursued research exploring the synthetic combination of several prominent classes of bioactive compounds to create new scaffolds with pharmacological potential. Pharmacophore hybridization has emerged as a paradigm for pharmaceutical chemists. The main motivations for using this strategy are the significant improvement in the therapeutic potential, strength, mode of action and pharmacokinetics [1-4]. Multicomponent reactions (MCRs) have recently been exploited as a means to synthesize a variety of chemically-hybridized compounds, including those of interest for biological evaluation. Among the synthetic methods utilized for this purpose, single-pot multi-component reactions provide the most efficient routes to the desired products, avoiding the need to isolate and purify intermediate products while improving atom economy and product yields [5]. Medicinal chemists have further exploited multi-component synthesis in order to construct compounds of pharmaceutical interest, by combining two or more structural subunits that have their own biological activities. In some cases, these biological activities are complementary to one another or aid in the enrichment of some desired properties, such as antimicrobial activity (bifunctional or even trifunctional prodrugs), or completely orthogonal (such as anticancer with antimicrobial). Recently our laboratories have explored applications of the MCR approach to uncover new chemical structures with intriguing biological properties. In this study, we explore the unique combination of two different classes of bioactive structures, namely fused chromenes and β-lactams (Figure 1).

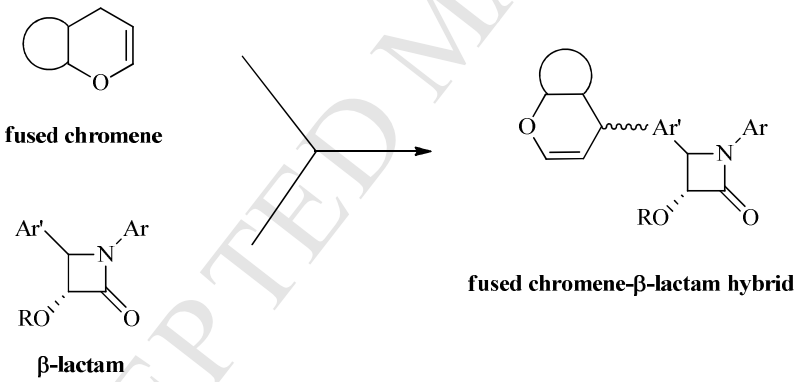


Fig. 1. Generalized structure of a fused chromene-β-lactam hybrid.

Tetrahydro-4*H*-chromenes and dihydropyrano[*c*]-chromenes have diverse pharmacological potential as anti-allergenic, anti-proliferative, antibacterial, antiviral, antifungal, antioxidant, antitumor, and anticancer agents [6-9]. Examples include benzopyrans **A** and **B**, which possess anticancer activity [10], and compound **C**, which has antibacterial activity [11] (Figure 2).

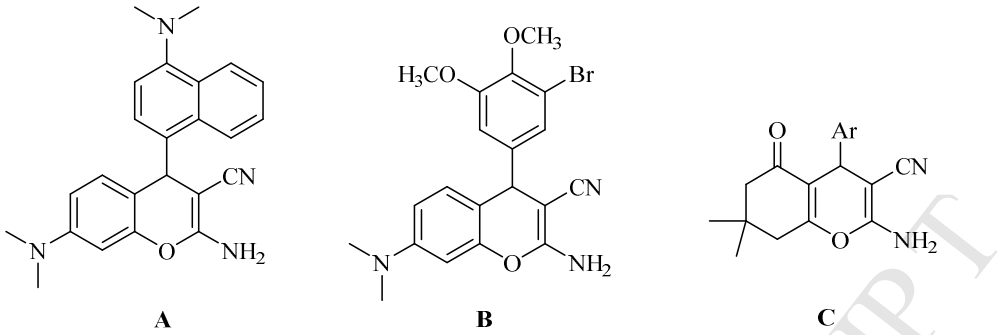


Fig. 2. Structures of biologically-active fused chromenes.

The discovery and clinical development of β-lactam antibiotics remains one of the major advances in modern medicine [12]. The commonly used bicyclic β-lactam antibiotics such as the penicillins and cephalosporins prevent bacterial transpeptidases from crosslinking the polysaccharide cell wall [13]. More recently, functionalized monocyclic β-lactams have been developed having a host of other pharmaceutical capabilities, such as anti-HIV [14], antifungal [15], anticancer [16,17] and antimalarial [18] (Figure 3), anti-inflammatory, or analgesic activities [19]. Thus, the β-lactam framework presents a rich source of diverse biological properties.

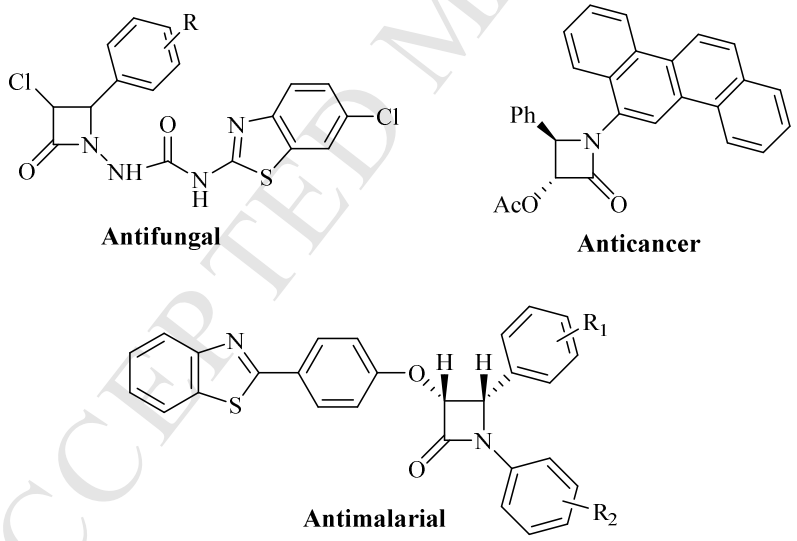
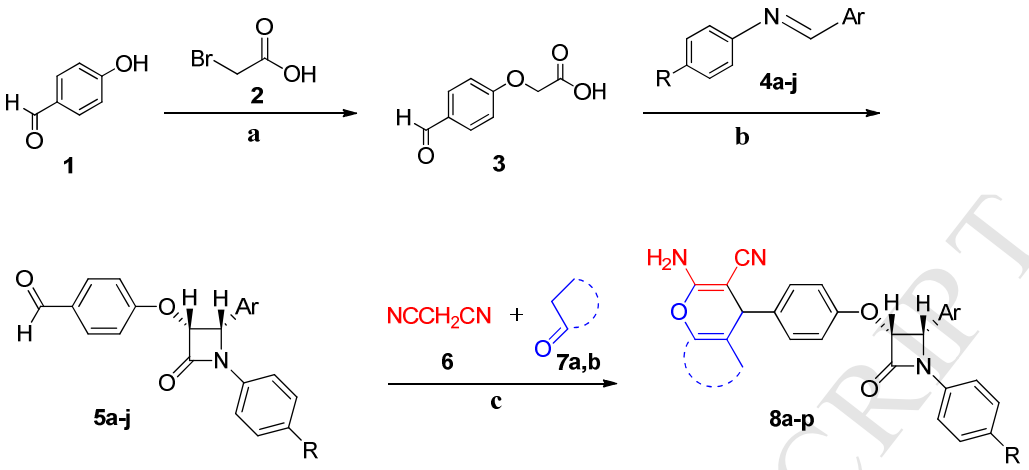


Fig. 3. Structures of some biologically active monocyclic β-lactam derivatives.

In this current study, we set our focus on structurally unique β-lactams **8a-p** bearing tetrahydro-4*H*-chromene and dihydropyrano[*c*]-chromene pharmacophores, through the development of a single-pot, multi-component coupling strategy illustrated in Scheme 1.



Reagents and conditions: (a) K_2CO_3/DMF/70 °C; (b) TsCl/Et_3N/CH_2Cl_2/r.t; (c) DABCO in refluxing EtOH (refer to Table 1 for identity of R and Ar.)

Scheme 1. Multi-component synthesis of aldehydic β-lactams **5a-j** and chromeno-β-lactam hybrids **8a-p**.

2. Results and discussion

2.1. Chemistry

In this study, β-lactams **5a-j** were synthesized by a diastereoselective ketene-imine cycloaddition reaction (Scheme 1) [18,20]. The reaction of 4-hydroxybenzaldehyde (**1**) with bromoacetic acid (**2**) in DMF at 70°C in the presence of solid K_2CO_3 afforded 2-(4-formylphenoxy)acetic acid (**3**). Treatment of **3** with Schiff bases **4a-j** in the presence of triethylamine and p-toluenesulfonyl chloride produced the *cis*-β-lactams **5a-j** in isolated yields varying from 68-80% after column chromatography (Table 1).

Table 1. Isolated yields of aldehydic β-lactam hybrids **5a-j**.

Cpd	R	Ar	Isolated yield (%)
5a	OMe	4-ClPh	80
5b	Me	4-ClPh	75
5c	Cl	3-NO_2Ph	77
5d	OMe	3-NO_2Ph	70
5e	OEt	4-ClPh	80
5f	OEt	4-NO_2Ph	80
5g	NMe_2	4-NO_2Ph	88
5h	OEt	4-NMe_2Ph	74
5i	OEt	anthracene	80
5j	OMe	anthracene	80

Structures of the cycloadducts **5a-j** were established on the basis of their IR, ^{1}H NMR, ^{13}C NMR spectral data and elemental analysis (see supporting information). The IR spectra of **5b** (R=Me, Ar=4-ClPh) showed absorption band at υ 1759 cm^{-1} for the β-lactam carbonyl group and absorption band at υ 1689 cm^{-1} for the aldehyde carbonyl group. Compound **5b** showed doublets at δ 5.79 ppm and δ 6.03 ppm for the H-2 and H-3 β-lactam ring protons. The aldehydic hydrogen resonated as a singlet at δ 9.81 ppm. The ^{13}C NMR spectral data for compound **5b** exhibited signals at δ 60.9 and δ 80.4 ppm for the C-2 and C-3 β-lactam ring protons, respectively, the aromatic carbons at appropriate shifts, the β-lactam carbonyl carbon at δ 161.4 ppm and the aldehyde carbonyl carbon at δ 190.5 ppm. The *cis* stereochemistry was assigned from the vicinal coupling constants of the two β-lactam ring protons, H-2 and H-3 ($J_{2,3}$ ≥4.0 Hz) [18]. Single crystal X-ray analysis of β-lactam **5b** confirmed the *cis* stereochemistry (Figure 4). Crystallographic data, details of the data collection and structure refinement can be found in the supplementary material [21].

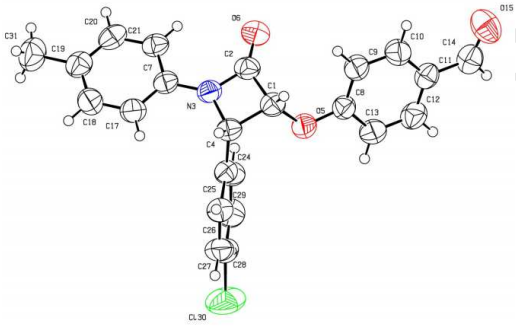
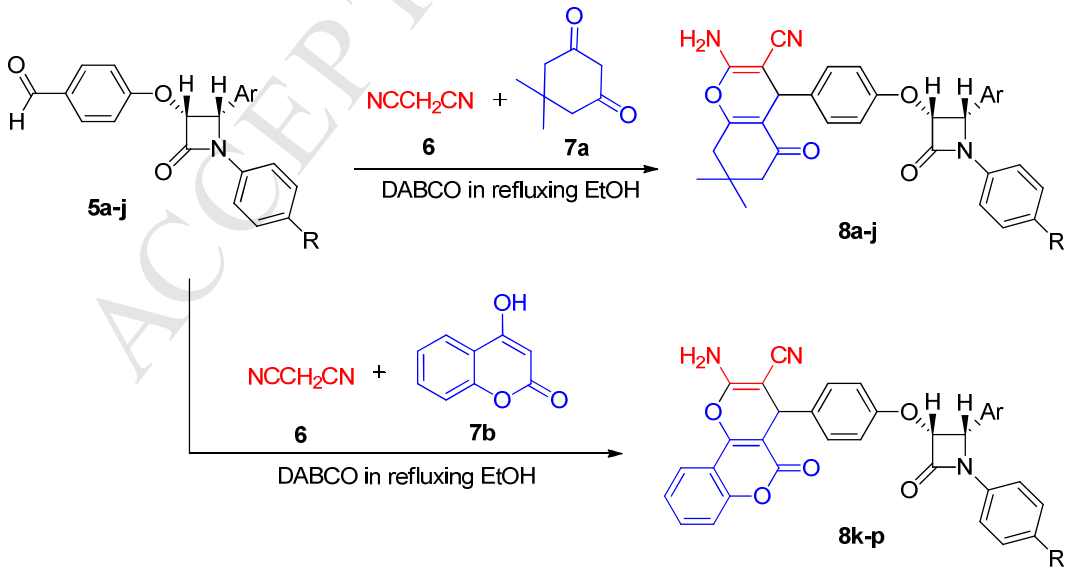


Fig. 4. ORTEP diagram of β-lactam **5b**.

From aldehydic β-lactams **5**, we further developed an expedient multi-component approach for constructing chromeno β-lactam hybrids **8** (Scheme 2).



Scheme 2. Multi-component synthesis of chromeno β-lactam hybrids **8a-p**.

To optimize the reaction conditions, β-lactam **5a** was stirred in ethanol with equimolar amounts of malononitrile (**6**) and 5,5-dimethylcyclohexane-1,3-dione (**7a**) in the presence of different base catalysts (Table 2). A higher yield and shorter reaction time were observed when the reaction was carried out in the presence of a molar equivalent of 1,4-diazabicyclo[2.2.2]octane (DABCO) under reflux conditions (entry 6) [22,23].

Table 2. Effects of reagent and solvent on the reaction of β-lactam **5a**, malononitrile (**6**) and 5,5-dimethylcyclohexane-1,3-dione (**7a**) under different reaction conditions.

Entry	Reagent	Reagent (mmol)	Solvent	Temp (^oC)	Time (h)	Yield
1	——	——	EtOH	Reflux	48	——
2	Et_3N	1.0	EtOH	Reflux	5	70
3	K_2CO_3	1.0	EtOH	Reflux	8	62
4	DABCO	0.5	EtOH	Reflux	1.5	75
5	DABCO	1.5	EtOH	Reflux	1.5	95
6	**DABCO**	**1.0**	**EtOH**	**Reflux**	**1.5**	**95**
7	DABCO	1.0	MeCN	Reflux	10	20
8	DABCO	1.0	H_2O	Reflux	10	50
9	DABCO	1.0	MeOH	Reflux	10	55
10	DABCO	1.0	$CHCl_3$	Reflux	10	Trace
11	DABCO	1.0	EtOAc	Reflux	10	24
12	DABCO	1.0	EtOH	r.t.	8	60
13	DABCO	1.0	——	80	10	33
14	DABCO	1.0	——	100	10	46

Subsequently, these optimized reaction conditions were used for the condensations of β-lactams **5a-j** (Table 3).

Table 3. Isolated yields of chromeno β-lactam hybrids **8a-p** under the optimized reaction conditions.

Cpd	R	Ar	Time (h)	Yield (%)	Cpd	R	Ar	Time (h)	Yield (%)
8a	OMe	4-ClPh	1.5	95	**8i**	OEt	Anthracene	1.5	92
8b	Me	4-ClPh	2.5	90	**8j**	OMe	Anthracene	1.5	90
8c	Cl	3-NO_2Ph	2.2	92	**8k**	OEt	4-ClPh	3.0	85
8d	OMe	3-NO_2Ph	3.5	80	**8l**	OMe	4-ClPh	2.6	92
8e	OEt	4-ClPh	2.5	94	**8m**	NMe_2	4-NO_2Ph	4.0	82
8f	OEt	4-NO_2Ph	1.6	95	**8n**	OEt	4-NMe_2Ph	3.8	90
8g	NMe_2	4-NO_2Ph	3.2	85	**8o**	OEt	Anthracene	1.8	95
8h	OEt	4-NMe_2Ph	3.0	90	**8p**	OMe	Anthracene	2.0	90

Structures of the chromeno β-lactam hybrids **8a-p** were established from their IR, ^{1}H NMR, ^{13}C NMR spectral data, H-H COSY and H-C HSQC two-dimensional NMR studies, and elemental analysis (see supporting information). The IR spectra showed the anticipated NH_2 stretching vibration, the CN stretching, and the β-lactam carbonyl signals in regions similar to those for the precursor β-lactams **5a-j**. We also noted the absence of the aldehyde carbonyl group at 1689 cm^{-1}, and the appearance of a sharp band at 1666 cm^{-1} for the vinylogous carbonyl (ketone or lactone). The ^{1}H NMR spectra showed doublets with J = 5.0 Hz for the two β-lactam ring protons, H-2 and H-3, and NH_2 protons in the aromatic region. For compound **8b** as a representative example, these NH_2 protons deuterium-exchanged with D_2O and the integration of 4Hs in the region of δ 6.88-6.95 (m, ArH, NH_2) decreased to 2H, producing a sharp doublet at δ 6.94 (J = 10.0 Hz). The ^{13}C NMR spectral data for each of the compounds exhibited carbonyl, aliphatic and aromatic carbons at appropriate chemical shifts. H-H COSY and H-C HSQC NMR experiments also corroborated the stereochemical assignments, in that the correlations observed in the two-dimensional spectra for lactams **8a**, **8b**, **8c**, **8f**, **8g**, **8i**, **8j**, **8l**, **8n**, **8o**, and **8p** all were in alignment with the structure having *cis*-disubstitution on the β-lactam rings.

2.2. Biological activities

2.2.1. Anti-inflammatory activity assay

Inflammation contributes to the degenerative processes of host of human ailments and diseases, such as cancer, arthritis, atherosclerosis, autoimmune diseases and aging of skin and other tissues and organs. Treatment with anti-inflammatory compounds may reduce the severity of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most popular pharmaceutical agents for the treatment of chronic inflammatory diseases, pain and fever that exert their therapeutic effect *via* blockade of cyclooxygenase isoenzymes (arachidonic acid pathway) to prevent the production of prostaglandins and other inflammatory mediators. Nitric oxide (NO) plays significant roles in the pathophysiology of inflammatory illnesses. It gives an anti-inflammatory effect under normal physiological conditions. NO is synthesized and released

into the endothelial cells by the help of nitric oxide synthases that convert arginine into citrulline producing NO in the process. Also, NO is considered as a pro-inflammatory mediator that induces inflammation because of over production in abnormal situations. Therefore, NO inhibitors represent important therapeutic advance in the management of inflammatory diseases [24]. In order to identify new anti-inflammatory candidates, the RAW 264.7 murine macrophage assay was used to examine anti-inflammatory behavior of the new β-lactam hybrids we synthesized. This assay monitors the inflammatory cascade leading to an overproduction of NO in the endothelial lining of blood vessels. In the present study, it was performed to evaluate the capacity of the compounds to inhibit the pro-inflammatory cascade leading to NO production in mouse macrophages. Results are reported in Table 4. Compound **8b** was the most active of all the chromeno β-lactam hybrids tested, with a 19.8 anti-inflammatory ratio.

Table 4. Anti-inflammatory activity of chromeno β-lactam hybrids **8a-p**.

Sample	IC_{50} NO release Sample (μM)	IC_{50} cell viability Sample (μM)	Anti-inflammatory ratio
8a	ND	ND	ND
8b	6.24 ± 1.28	123.47 ± 13.24	19.8
8c	53.41 ± 5.13	124.21 ± 8.23	2.3
8d	100.02 ± 6.21	114.31 ± 10.31	1.1
8e	73.36 ± 7.94	201.45 ± 11.56	2.7
8f	89.42 ± 6.81	124.31 ± 11.39	1.4
8g	60.08 ± 5.24	251.48 ± 8.37	4.1
8h	38.10 ± 4.31	146.25 ± 7.23	3.8
8i	101.65 ± 23.24	165.23 ± 10.87	1.6
8j	56.41 ± 5.64	134.63 ± 8.24	2.4
8k	ND	ND	ND
8l	89.56 ± 3.81	124.31 ± 8.33	1.4
8m	161.36 ± 21.54	163.24 ± 11.42	1.0
8n	ND	ND	ND
8o	168.23 ± 11.41	224.34 ± 11.84	1.3
8p	164.57 ± 8.84	198.56 ± 12.09	1.2
Dexamethasone	5.02 ± 1.34 μM	159.2 ± 26.35 μM	31.9

ND: Not determined due to poor solubility

For comparison, we also checked anti-inflammatory activity for the aldehydic β-lactams **5a-j** (Table 5). As observed for lactam **8b**, the *p*-tolyl group on N-1 of the β-lactam ring of **5b** enhances anti-inflammatory activity in this assay, more active than the corticosteroid drug, dexamethasone.

Table 5. Anti-inflammatory activity of derivatives **5a-j**.

Sample	IC_{50} -NO release Sample (μM)	IC_{50}-cell viability Sample (μM)	Anti-inflammatory ratio
5a	92.42 ± 6.81	131.31 ± 9.39	1.4
5b	2.31 ±0.56	123.78 ± 7.49	53.4
5c	63.65 ± 4.07	132.48 ± 6.37	2.1
5d	2.91 ± 0.84	4.15 ± 1.66	1.4
5e	11.32 ± 0.89	56.28 ± 6.31	4.9
5f	97.73 ± 9.95	189.65 ± 8.18	1.9
5g	32.86 ± 3.35	116.84 ± 14.58	3.6
5h	125.20 ± 8.15	241.78 ± 11.42	1.9
5i	42.58 ± 4.27	112.56 ± 10.36	2.6
5j	69.11 ± 19.45	68.32 ± 6.87	1.0
Dexamethasone	5.02 ± 1.34 μM	159.2 ± 26.35 μM	31.9

2.2.2. Anticancer activity and cytotoxicity assays

In vitro cytotoxicity and anti-cancer activity were also evaluated for each of the compounds synthesized. Three of the chromeno β-lactam hybrid compounds (**8c**, **8j** and **8p**) showed elevated anticancer activity against the *SW1116* (colon cancer) cell lines, with IC_{50} values of 7.29, 8.83, and 7.43 μM, respectively, in comparison to the anticancer agent methotrexate (IC_{50} of 2.49 μM). The remainder of the lactams **8** afford no anticancer activity at or below 200 μM. Since all of these lactams carry a tetrahydro-4*H*-chromene or dihydropyrano[*c*]-chromene group on C-3 of the β-lactam ring, the aryl substituents at N-1 and C-2 of the β-lactam ring are responsible for these differences in activity. A tentative structure-activity relationship exists, in that the presence of a *p*-nitrophenyl or *p*-chlorophenyl group on C-2 of the β-lactam ring and a *p*-methoxyphenyl, *p*-tolyl, *p*-ethoxyphenyl or *p*-*N*,*N*-dimethylaminophenyl ring on N-1 of the β-lactam provide the best anticancer bioactivity. Additionally, five of the aldehydic β-lactams (**5a**, **5b**, **5e**, **5f** and **5g**) possess comparable IC_{50} values. IC_{50} values are 10.26, 10.00, 6.71, 8.19, and 6.93 μM, respectively. Conversely, none of the lactams (**5a-j** or **8a-p**) have cytotoxicity against *HepG2* cells at or below 200 μM (Table 6).

Table 6. Anticancer and cytotoxic activity assays of selected β-lactams **5** and **8**, assessed by the MTT reduction assay against *SW1116* and *HepG2* cells.

Compound	SW1116 IC_{50} (µM)	HepG2 IC_{50} (µM)
5a	**10.26**	**> 200**
5b	**10.00**	**> 200**
5e	**6.71**	**> 200**
5f	**8.19**	**> 200**
5g	**6.93**	**> 200**
8c	**7.29**	**> 200**
8j	**8.83**	**> 200**
8p	**7.43**	**> 200**
Methotrexate	**2.49**	**0.30**

2.2.3. Molecular docking studies

2.2.3.1. Validation of molecular docking

The performance of a common computational docking technique can be tested by checking its power to foresee the most significant binding mode of a cognate (co-crystallographic) ligand [25]. This method was carried out by eliminating the structure of a cognate ligand and re-docking it into its protein (self-docking). Root-mean-square deviation (RMSD) of the Cartesian coordinates of the atoms of the ligand in the docked and crystallographic conformations is the principle of the docking validation (RMSD < 2 Å). Validation of molecular docking showed RMSD values were for PDB ID: 4NOS = 0.4 Å and for PDB ID: 3LN1 = 0.01 Å (Supplementary Information).

2.2.3.2. Study of the binding mode

Molecular docking experiments suggest that chromeno β-lactams **8a-p** may bind to human inducible nitric oxide synthase protein *via* hydrogen bond and hydrophobic interactions with specific active site amino acid residues. The integration of computational and experimental techniques is an acutely attractive strategy for the design and optimization of drug candidates. Molecular docking studies showed that synthesized compounds **5a-j** and **8a-p** could inhibit human inducible nitric oxide synthase and cyclooxygenase-2 via hydrogen bond and hydrophobic interactions (Tables 7 and 8). According to molecular docking results, all compounds exhibited higher free binding energy values with human inducible nitric oxide synthase enzyme compared with cyclooxygenase-2. Therefore, these derivatives showed anti-inflammatory activity through inhibition of release of nitric oxide. Thus, we focused on

investigation of molecular docking results and binding modes of synthesized compounds at the human inducible nitric oxide synthase active site.

Table 7. Docking results of **5a-j** and **8a-p** derivatives docked into the human inducible nitric oxide synthase target (PDB ID: 4NOS).

Code	$\Delta G_{binding}$ (Kcal/mol)	Hydrogen bond	Hydrophobic interaction	π-π	Cation-π
5a	-7.54	-	Gly371, Ala351, Pro350, Asn370, Val352, Glu377, Asp382, Arg266, Trp346, Tyr347, Gln263, Ala262, Asn354	-	-
5b	-10.30	Gln263	Ala351, Asp382, Arg381, Glu377, Tyr373, Pro350, Ala262, Val352, Met355, Asn354	-	-
5c	-9.30	Cys110	Gly117, Leu116, Ser118, Cys115, Ile462, Gln478, Trp461, Glu479	Trp461	-
5d	-9.23	Cys110	Gln478, Trp461, Glu479, Ile462, Gly117, Cys115, Ser118, Leu116	-	-
5e	-9.91	Arg388, Tyr373	Arg266, Arg388, Ala262, Val352, Gln263, Pro350, Gly371, Asn370, Phe369, Glu377	-	Arg266
5f	-8.35	Tyr373, Arg388	Asp385, Arg381, Ala282, Arg266, Asn354, Ala262, Gln263, Met355, Val352, Pro350, Glu377, Trp372, Gly371	Tyr491	-

5g	-9.65	Cys110	His477, Gln478, Glu479, Met480, Phe476, Trp461, Cys115, Ser118, Ile462, Gly117	-	-
5h	-8.19	Tyr373, Arg388	Asp382, Glu377, Gln263, Val352, Ala351, Phe369, Pro350, Gly371, Asn370	Hem	-
5i	-8.64	-	Trp461, Pro466, Ile462, Val465, Pro467, Asp385, Arg381, Trp463	Hem	Arg381
5j	-8.53	-	Trp461, Ile462, Pro466, Val465, Trp463, Pro467, Arg381, Asp385	Hem	Arg381
8a	-9.23	Arg381, Asp382	Gln263, Arg266, Arg388, Glu377, Val352, Met120, Trp463, Pro466, Pro467, Ile462	Hem	-
8b	-10.01	Arg381, Asp382	Gln263, Val352, Glu377, Arg388, Trp463, Met120, Pro467, Ile462, Pro466, Val465	-	-
8c	-8.80	Gly470, Trp463	Met120, Arg381, Ile462, Pro467, Val465, Pro466, Trp461, Phe476, Ser469, Met468	-	-
8d	-8.82	Arg381	Val352, Glu377, Gln263, Arg388, Met120, Val465, Trp463, Pro466, Pro467, Ile462	-	-
8e	-9.18	-	Asp412, Cys384, Leu392, Lys411,	-	-

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			Val386, Val415, Pro467, Val380, Arg381, Asp385, Val465, Pro466, Ile462, Trp461		
8f	-8.99	Arg388, Tyr373	Asp385, Arg381, Ala282, Arg266, Asn354, Ala262, Met355, Val352, Gln263, Gly371, Pro350, Trp372, Glu377	-	Arg266
8g	-9.71	-	Ala351, Glu377, Val352, Gln263, Arg266, Tyr491, Met355, Asn354, Ala262, Gln492, Glu494, Lys497, Thr121	Hem	-
8h	-9.13	Gly371, Tyr373, Gln263	Arg388, Met120, Thr121, Asn354, Ala262, Met355, Trp463, Val352, Glu377, Ala351, Phe369, Pro350, Asn370	-	-
8i	-8.52	Lys411	Val465, Trp463, Met120, Arg381, Ile462, Pro467, Met468, Val415, Val380, Asp385, Cys384	-	Arg381
8j	-9.16	-	Met120, Arg381, Ile462, Pro466, Trp463, Val465, Pro467, Asp385, Cys384, Val380, Val415, Lys411, Met468	-	Arg381
8k	-9.61	Arg381, Asp382	Ala351, Pro350, Val352, Pro467, Gln263, Arg388,	-	-

			Trp463, Met120, Ile462		
8l	-8.79	Tyr373, Arg388, Arg381	Arg266, Glu377, Met120, Ala282, Asn354, Val352, Gln263, Ala262, Pro350, Gly371, Asn370	Hem	Arg266
8m	-8.53	Arg381	Pro350, Ala351, Arg388, Val352, Met374, Gln263, Arg266, Pro466, Pro467, Val465, Met120, Trp463, Ile462	Hem	-
8n	-8.49	Arg381	Met355, Asn354, Ala262, Tyr491, Arg266, Val352, Gln263, Met120, Glu377, Met374, Ile462, Trp461, Val465, Trp463, Pro467	Tyr491 , Hem	-
8o	-9.44	Gln263	Glu285, Ala282, Gln387, Ala262, Asn354, Phe369, Tyr491, Val352, Met120, Trp463	-	-
8p	-9.39	Arg381	Val352, Tyr491, Met355, Asn354, Trp463, Met120, Pro467, Ile462	Hem	-
Dexamethasone	-8.01	Tyr373, Arg388, Gln263, Glu377	Asp382, Val352, Phe369, Asn370, Pro350, Gly371	-	-

Table 8. Docking results of **5a-j** and **8a-p** derivatives docked into the cyclooxygenase-2 target (PDB ID: 3LN1).

Code	$\Delta G_{binding}$ (Kcal/mol)	Hydrogen bond	Hydrophobic interaction	π-π	Cation-π
5a	-8.43	Arg499	Tyr371, Phe367, Leu370, Gly512, Ser516, Trp373, Ala513, Ser339, Val335, Phe504, Val509, Leu338, Ala502, His75, Tyr341	Phe504, Trp373, Tyr371	-
5b	-9.53	Arg106	Leu370, Gly512, Met508, Trp373, Val509, Val335, Phe504, Ala502, Leu338, Ser339, Tyr341, Leu345, Leu517, His75, Ala513, Val102	-	-
5c	-9.54	-	Trp373, Phe367, Gly512, Leu370, Tyr371, Ser516, Val509, Ala513, Leu338, Phe504, Leu345, His75, Ser339, Val335, Tyr341, Val102, Arg106, Ala502, Arg499	Phe504, Trp373	-
5d	-9.26	Tyr371, Tyr341, Arg106, His75	Gln178, Ser339, Val509, Ala502, Leu338, Ala513, Phe504, Gly512, Ser516, Val335, Tyr334, Val330	Phe504	-
5e	-9.18	Arg499	Ser339, Val509, Tyr341, His75, Ala502, Phe504,	Tyr371	-

			Ala513, Leu338, Leu517, Leu520, Val335, Tyr334, Tyr371, Ser516, Phe191, Phe367		
5f	-8.61	-	His75, Ala502, Tyr341, Ser339, Gln178, Val509, Leu338, Phe504, Tyr371, Leu517, Val102, Ser516, Leu345, Ala513, Val335, Tyr334, Gly512, Phe367, Leu520, Phe191	Tyr371	-
5g	-8.35	-	Leu370, Val509, Met508, Trp373, Phe504, Ser516, Leu338, Gly512, Val335, Ser339, Ala513, Leu517, Tyr341, Arg106, Leu345, Val102	-	-
5h	-8.82	Arg499	His75, Ser339, Tyr341, Val509, Phe504, Ala502, Ala513, Leu338, Tyr371, Val102, Leu345, Val335, Leu517, Ser516, Phe367, Tyr334, Phe191	Tyr371	-
5i	-7.25	His75	Pro500, Thr79, Asp501, Gln178, Gln336, Gly340, Tyr341, His337, Pro177, His342, Val568, Asn567	His342	-
5j	-7.11	-	Pro500, Ala502, Thr79, His75, Gly340, Pro177, Gln178, Asp501, His337, Tyr341,	His342	-

			His342, Gln336, Val568, Asn567, Gly179		
8a	-6.14	-	Pro500, Arg499, Ala502, Thr79, Asp501, Gly340, His75, His342, Gln336, Ser339, Gln178, Asp333, Asn567, His337	His342	-
8b	-6.07	Gln336, Thr79	Arg499, Ala502, His75, Pro500, Gln178, Ser339, Tyr341, Gly340, Asp501, His337, Asp333, Glu332, Asn567	-	-
8c	-6.59	Gln336, Thr79	Pro500, Asp501, Arg499, His75, Ser339, Ala502, His342, Gly340, Gln178, Ser565, His337, Asn567	-	-
8d	-6.31	Gln336	His75, Pro500, Gln178, Thr79, Asp501, Gly340, Ser339, Ala502, Tyr341, His337, Asn567, Asp333, Glu332	-	-
8e	-5.85	His75, Gln178	Gly179, Asn567, His342, Pro177, Pro500, Gly340, Asp501, Ala502, Thr79, Tyr76, His80	His80	-
8f	-6.79	Gln178	Asn567, Gly340, Gly179, Pro177, His342, Pro500, Asp501, Ala502, Thr79, His80, His75, Tyr76	-	-

8g	-5.64	Gln336, Thr79	His75, Pro500, His342, Asp501, Ala502, Arg499, Gly340, His337, Gly179, Gln178, Asn567, Asp333	-	-
8h	-5.65	Pro500	Thr79, Ala502, Asp501, His342, Gly340, Ser339, His337, Gln178, Val568, Phe566, Asp333, Ile550, Ser565, Asn567, Gln336, Gly179	-	-
8i	-1.59	Gln178	His337, Asn567, Gln336, Gly340, Ser339, Thr79, Tyr76, Asn72, Ala502, His75, Arg499, His80, Pro500	-	-
8j	-5.45	Thr79	His75, Ala502, Thr79, Gln178, Pro500, Gly340, Asp501, Ser339, His80, Gln336, Phe566, Asp333, His337, Asn567	-	-
8k	-7.18	Gly340	Thr79, His80, Pro500, His75, His342, Asp501, His337, Gln178, Gln336, Asn567	-	-
8l	-7.26	Gln336, Gly340, Thr79	His75, His80, Pro500, Asp501, His342, Gln178, Asn567, His337, Asp333	-	-
8m	-5.37	Gln178	Asn567, Leu338, Gln336, His337, Arg499, Ser339, Gly179, Gly340,	-	-

			His75, Ala502, Pro500, Thr79, Asn72, Tyr56, His80		
8n	-5.53	Thr79	His80, Pro500, His75, His342, Asp501, Gly340, His337, Gln336, Gln178, Val568, Asn567, Gly179, Asp333, Ile550	His75	-
8o	-7.19	-	Pro500, His75, Thr79, Gln178, Ala502, Asp501, Gly340, Asn567, Phe566, His337, Asp333, Ile550, Gln336, His342, Tyr341	-	-
8p	-5.86	-	Asp501, Thr79, His75, His80, Pro500, Ala502, Lys82, Gly340, Asn567, Gln569, Val568, His337, Gln336, Gln178	-	Lys82
Dexamethasone	-7.23	Tyr373, Glu377	Asp382, Val352, Phe369, Asn370, Pro350, Gly371	-	-

Members of this group exhibit weak to good *in vitro* anti-inflammatory activity, such that higher lipophilicity enhances these particular hydrophobic interactions. Indeed, **8b** exhibited the best docking score from the computational studies, coinciding with the most potent *in vitro* anti-inflammatory activity. Interactions of **8b** with the enzyme active site are depicted in Figure 5. The carbonyl and 4-oxyphenyl oxygen atoms interact through hydrogen bonds with Arg381 (O…NH, 1.96 Å and O…NH, 1.98 Å). Also the amine nitrogen atom of the C-2 chromeno moiety is involved in a hydrogen bond with Asp382 amino acid (NH…O, 1.96 Å). Compound **8b** was found to also interact with a hydrophobic surface formed by Gln263, Val352, Glu377, Arg388, Trp463, Met120, Pro467, Ile462, Pro466 and Val465 residues.

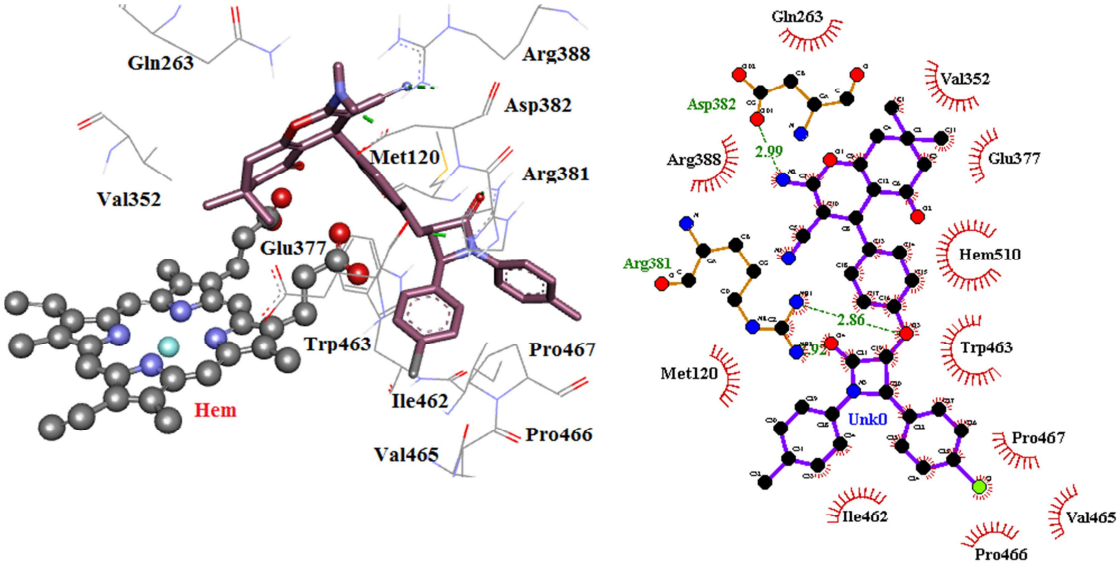

Fig. 5: Compound **8b** in the human inducible nitric oxide synthase active site. The 3D (left) and 2D (right) ligand interaction diagrams.

Replacement of the *p*-chlorophenyl group at C-4 position of the lactam ring in **8e** with a *p*-nitrophenyl in **8f** substantially decreased the *in vitro* activity. The calculated CLogP value for **8e** (6.75) indicates that it is somewhat more lipophilic than **8f** (CLogP = 5.78) and can thus experience stronger interactions with hydrophobic amino acids, accounting for its better anti-inflammatory activity and free binding energy (Table 4 & 7).

Replacing the *p*-(*N*,*N*-dimethylamino)phenyl ring of **8h** with an anthracene moiety at the C-4 position (**8i**) decreased the *in vitro* activity. The lower anti-inflammatory activity of **8i** can be attributed to steric hindrance and a change in the orientation of compound **8i** in the active site, which weakens the intermolecular interactions.

Dexamethasone was likewise computationally docked into the protein and showed a similar binding mode to the β-lactams, forming hydrogen bonds and hydrophobic interactions with key residues in the enzyme active site. (Figure 6).

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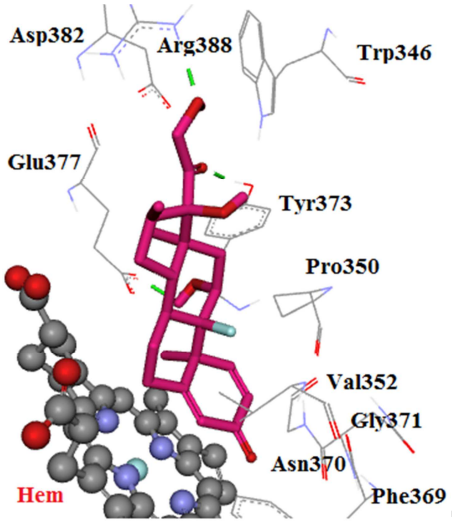

Fig. 6: Hydrogen bonds and hydrophobic interactions of dexamethasone within the human inducible nitric oxide synthase active site.

For comparison, we also examined the docking of aldehydic β-lactams **5** into the human inducible nitric oxide synthase active site. Compounds **5b** and **5e** gave the best docking scores that correspond with their strong anti-inflammatory activity (Table 5). The carbonyl oxygen atom of lactam ring of **5b** formed a hydrogen bond interaction with NH of Gln263 (O…HN, 1.84 Å). Moreover, **5e** formed two hydrogen bonds with amino acid active sites. The carbonyl oxygen atom of lactam ring displayed a hydrogen bond with OH group of Tyr373 (O…HO, 2.59 Å) and the carbonyl oxygen atom of aldehyde showed a hydrogen bond with NH group of Arg388 (O…HN, 2.18 Å). The binding mode of compounds **5b** and **5e** to the human inducible nitric oxide synthase active site is revealed in Figure 7.

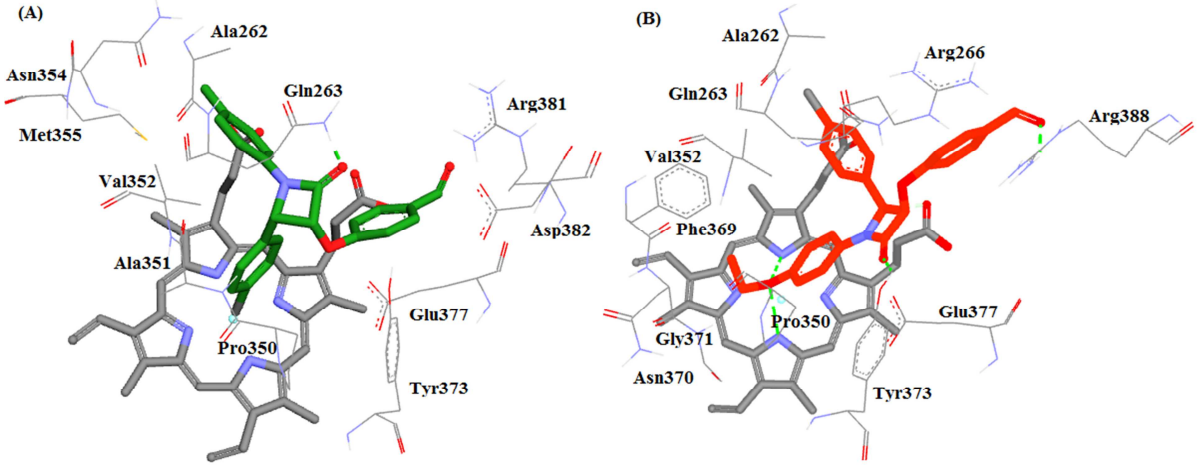

Fig. 7: Binding modes of compound **5b (A)** and **5e (B)** with human inducible nitric oxide synthase active site (PDB ID: 4NOS).

The three aryl rings of **5b** were located within the hydrophobic pocket enclosed by Ala351, Asp382, Arg381, Glu377, Tyr373, Pro350, Ala262, Val352, Met355 and Asn354. Exposed

residues Arg266, Arg388, Ala262, Val352, Gln263, Pro350, Gly371, Asn370, Glu377 and Phe369 also formed hydrophobic interactions with **5e**. Docking calculations revealed that **5e** forms a cation-π interaction with the Arg266 residue. All these interactions help anchor **5b** and **5e** in the binding site of the human inducible nitric oxide synthase protein. In addition, anti-inflammatory activity of **5b** was higher than dexamethasone as positive control ($IC_{50\text{-NO release}}$ = 5.02 μM).

Replacing the 4-chlorophenyl ring (**5e**) with the 4-nitrophenyl ring (**5f**) and 4-(*N*,*N*-dimethylamino)phenyl ring (**5h**) decreased the anti-inflammatory activity. The increased polarization and strong electron-donating characters reduced the potency of compounds but addition of hydrophobic groups had a positive effect. Also, docking results exposed that compounds **5e** and **5f** formed a cation-π and π-π stacking interactions with Arg266 and Tyr491 amino acids, respectively. While compound **5h** did not participate in these contacts (Figure 8).

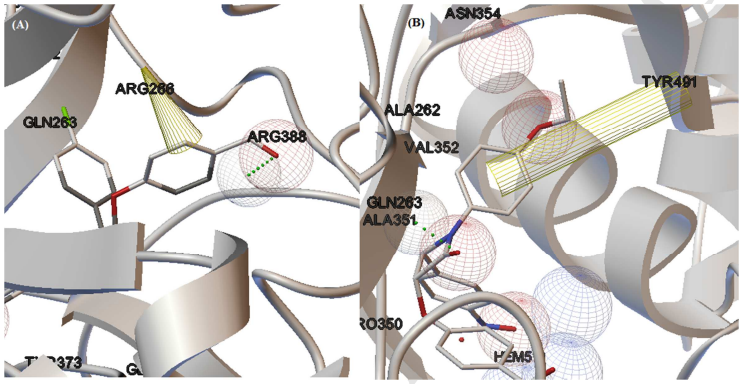

Fig. 8: Close up of the calculated binding of **5e (A)** and **5f (B)** within the human inducible nitric oxide synthase enzyme active site.

A methoxy or methyl group at the *para* position of the aryl ring induced different conformations, leading to a change in interactions with the hydrophobic pocket of the protein. This suggests that this is the reason for compound **5b** showing higher anti-inflammatory activity and ΔG_{bind} value than compound **5a**. In addition, the carbonyl oxygen atom of lactam ring of **5b** formed a hydrogen bond interaction with NH of Gln263 (O…HN, 1.84 Å), whereas **5a** did not have this interaction. The superimposition of **5a** and **5b** in the enzyme binding site is illustrated in Figure 9.

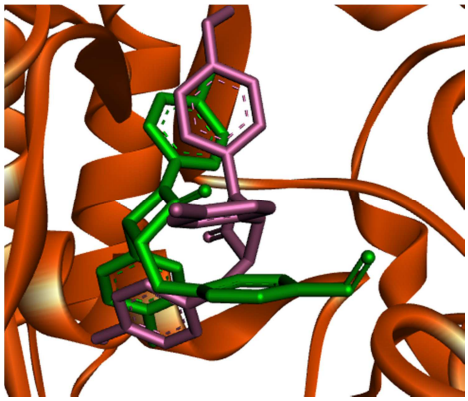

Fig. 9: Superimposition of **5a** (violet) and **5b** (green) within the enzyme binding site.

Compound **5a,** having a methoxy group at the *para* position of the aryl ring, showed lower inhibitory activity than compound **5e** that has an ethoxy group at the same position (Table 5). The lower anti-inflammatory activity of **5a** can be attributed to the improper geometric orientation in the active site of enzyme. Also, compound **5e** shows two hydrogen bonds with Arg388 and Tyr373 residues and a cation-π stacking interaction with Arg266, while compound **5a** did not have these interactions (Figure 10). Docking calculations indicate that **5e** has stronger interactions than **5a** with active site amino acids, accounting for its elevated anti-inflammatory activity.

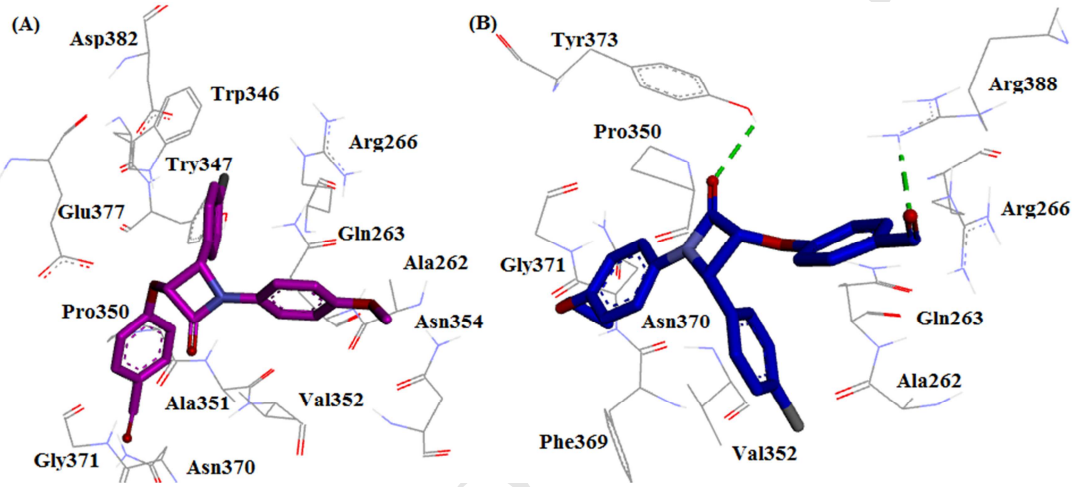

Fig. 10: Calculated binding modes of compound **5a (A)** and **5e (B)** within the human inducible nitric oxide synthase active site (PDB ID: 4NOS).

It is also interesting to compare β-lactam **5e** with **5i**. While **5e** has a *p*-chlorophenyl ring at the C-2 position of lactam ring, **5i** has a bulkier anthracene moiety (Figure 11). The computational modeling suggests that steric differences between these two aryl groups, along with the differences in the calculated ΔG_{bind} values for lactams **5e** and **5i**, may account for the different anti-inflammatory potencies.

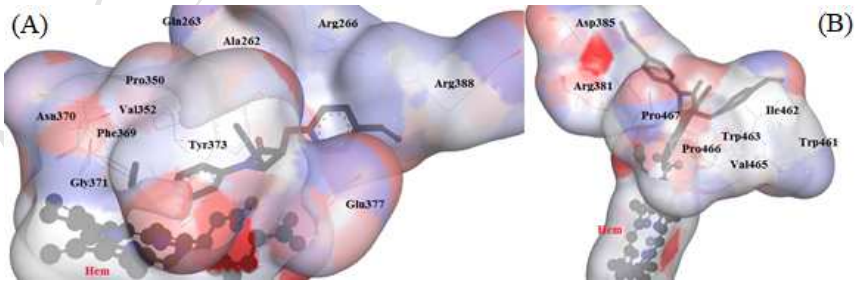

Fig. 11: Calculated binding mode of compound **5e (A)** and **5i (B)** within the human inducible nitric oxide synthase active site (PDB ID: 4NOS).

Replacing the *N*-aryl ethoxy substituent (**5f**) with an *N*,*N*-dimethylamino (**5g**) increased anti-

inflammatory activity, suggesting that increased electron-donation into the N-aryl ring improves potency. This is corroborated by computational docking experiments showing that while compound **5f** forms looser hydrogen bonds with Tyr373 and Arg388, compound **5g** forms a tighter hydrogen bond to the Cys110 residue in the enzyme binding pocket.

3. Conclusion

This describes the first diastereoselective synthesis of chromeno-substituted β-lactams. The compounds were accessed by an expedient single-pot three-component synthesis approach, to give exclusively the *cis*-disubstituted adducts. The series of chromeno β-lactam hybrids **8a-p**, as well as their aldehydic precursors **5a-j**, were evaluated for anti-inflammatory activity. The best anti-inflammatory activity was observed for compounds **5b** and **8b** (53.4 and 19.8 anti-inflammatory ratio, respectively). Moreover, based on the results of anti-inflammatory activity and molecular docking calculations of the synthesized compounds, the anti-inflammatory activity is closely dependent on the substituents at the N1 and C-2 centers of the lactam ring. Chromeno lactams **8c**, **8j** and **8p,** as well as aldehydic lactams **5a**, **5b**, **5e**, **5f**, **5g**, display *in vitro* anticancer activity against the *SW1116* colon cancer cell line, without noticeable cytotoxicity towards healthy cells. Studies are now underway to improve the biological activity and determine the mechanism of action.

4. Experimental Section

4.1 General experimental methods

All needed chemicals were purchased from Fluka, Aldrich, Merck and Across chemical companies and used without further purification. Solvents and reagents that used such as CH_2Cl_2 and Et_3N were dried before to use by distillation over CaH_2. All products were characterized by comparison of FT-IR 8300 spectrophotometer using potassium bromide pellets (υ in cm^{-1}). The ^1H NMR (250 MHz) and ^{13}C NMR (100 MHz) spectra, as well as the two-dimensional H-H COSY and H-C HSQC experiments, were run on a BrukerAvance400 in $CDCl_3$ using a Bruker Avance DPX instrument. Chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane. Coupling constants (J) are reported in hertz (Hz). Splitting patterns are indicated as s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, dd: doublet of doublet. Melting points were recorded on a Buchi 510 melting point apparatus in open capillary tubes. Elemental analyses were run on a Thermo Finnigan Flash EA-1112 series. The mass spectra were recorded on a Shimadzu GC-MS QP 1000 EX instrument. Thin-layer chromatography (TLC) was carried out on silica gel 254. X-ray data were collected on a Bruker D8 VENTURE diffractometer.

4.2. General procedure for the synthesis of some new β-lactams containing benzaldehydes 5a-j:

To a solution of 4-hydroxybenzaldehyde (**1)** (1.00 mmol) in 5 mL DMF in a 250 mL beaker, was added bromoacetic acid (1.50 mmol) (**2)** and potassium carbonate (5.00 mmol). The mixture was stirred at room temperature for 24 h. Then the reaction mixture was poured into 20 mL water with stirring. The solution was adjusted to pH 2.0 with 2.0M hydrochloric acid, where upon precipitation occurred. The product was collected and washed with water (20 mL). The crude

product was purified by recrystallization from ethanol to give 2-(4-formylphenoxy)acetic acid (**3)**. A solution of corresponding imine **4a-j** (1.00 mmol) was stirred with the corresponding substituted 2-(4-formylphenoxy)acetic acid (**3)** (1.50 mmol), *p*-toluenesulfonyl chloride (0.69 mL, 1.5 mmol) and triethylamine (5.00 mmol) in dry CH_2Cl_2 at room temperature overnight. Then it was washed with HCl 1.0 N (20 mL), saturated $NaHCO_3$ (20 mL), brine (20 mL), dried over Na_2SO_4 and the solvent was evaporated under reduced pressure to give the crude product. It was then purified by recrystallization from ethanol to afford pure β-lactams containing benzaldehydes **5a-j**.

4.2.1. 4-((2-(4-Chlorophenyl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5a**):

White solid; Mp. 175-177 °C; IR (KBr, cm^{-1}): 1766 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 3.68 (3H, s, OCH_3), 5.77 (1H, d, *J* = 4.0 Hz, H-2 β-lactam), 6.02 (1H, d, *J* = 4.0 Hz, H-3 β-lactam), 6.91 (2H, d, *J* = 8.2 Hz, ArH), 7.02 (2H, d, *J* = 8.0 Hz, ArH), 7.22 (2H, d, *J* = 8.2 Hz, ArH), 7.30 (2H, d, *J* = 7.7 Hz , ArH), 7.37 (2H, d, *J* = 8.0 Hz , ArH), 7.75 (2H, d, *J* = 8.0 Hz , ArH), 9.81 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.3 (CO aldehyde), 161.1 (CO β-lactam), 160.7, 156.1, 132.3, 131.4, 131.1, 130.6, 130.2, 129.7, 121.7, 118.5, 115.4, 114.6 (aromatic carbons), 80.1 (C-3 β-lactam), 59.9 (C-2 β-lactam) , 55.2 (O-CH_3); GC-MS m/z = 407 [M^+]; Analysis calculated for $C_{23}H_{18}ClNO_4$: C, 67.73; H, 4.45; N, 3.43%. Found: C, 66.18; H, 4.35; N, 3.62%.

4.2.2. 4-((2-(4-Chlorophenyl)-4-oxo-1-(p-tolyl)azetidin-3-yl)oxy)benzaldehyde (**5b**):

White solid; Mp. 178-180 °C; IR (KBr, cm^{-1}): 1759 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 2.21 (3H, s, CH_3), 5.79 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 6.03 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 7.02 (2H, d, *J* = 8.7 Hz, ArH), 7.13 (2H, d, *J* = 8.7 Hz, ArH), 7.18 (2H, d, *J* = 8.2 Hz, ArH), 7.29 (2H, d, *J* = 8.7 Hz, ArH), 7.36 (2H, d, *J* = 8.5 Hz, ArH), 7.75 (2H, d, *J* = 8.7 Hz , ArH), 9.81 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 190.5 (CO aldehyde), 161.4 (CO β-lactam), 159.0, 131.6, 131.1, 130.7, 129.7, 129.3, 128.8, 122.7, 120.9, 119.9, 117.4, 115.6 (aromatic carbons), 80.4 (C-3 β-lactam), 60.9 (C-2 β-lactam), 20.9 (CH_3); GC-MS m/z = 391 [M^+]; Analysis calculated for $C_{23}H_{18}ClNO_3$: C, 70.50; H, 4.63; N, 3.57%. Found: C, 70.33; H, 4.31; N, 3.61%.

4.2.3. 4-((1-(4-Chlorophenyl)-2-(3-nitrophenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5c**):

White solid; Mp. 232-234 °C; IR (KBr, cm^{-1}): 1751 (CO β-lactam), 1697 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 6.02 (1H, d, *J* = 5.0 Hz, H-2 β-lactam), 6.15 (1H, d, *J* = 5.0 Hz, H-3 β-lactam), 7.01 (2H, d, *J* = 8.5 Hz, ArH), 7.34 (2H, d, *J* = 8.7 Hz, ArH), 7.42 (2H, d, *J* = 9.0 Hz, ArH) 7.55 (1H, t, *J* = 8.0 Hz, ArH), 7.73 (2H, d, *J* = 8.5 Hz, ArH), 7.80 (1H, d, *J* = 7.7 Hz, ArH), 8.08 (1H, d, *J* = 8.2 Hz, ArH), 8.23 (1H, s, ArH), 9.79 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 190.2 (CO aldehyde), 161.4 (CO β-lactam), 160.6, 147.9, 134.6, 134.3, 133.7, 131.4, 130.9, 129.9, 129.5, 129.2, 123.7, 122.9, 118.4, 115.3 (aromatic carbons) , 80.4 (C-3 β-lactam), 60.2 (C-2 β-lactam); GC-MS m/z = 422 [M^+]; Analysis calculated for $C_{22}H_{15}ClN_2O_5$: C, 62.50; H, 3.58; N, 6.63%. Found: C, 61.93; H, 3.45; N, 6.80%.

4.2.4. 4-((1-(4-Methoxyphenyl)-2-(3-nitrophenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5d**):

Cream solid; Mp. 205-207 °C; IR (KBr, cm^{-1}): 1751 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 3.68 (3H, s, OCH_3), 5.97 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 6.11 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.91 (2H, d, *J* = 8.7 Hz, ArH), 7.01 (2H, d, *J* = 8.5 Hz, ArH), 7.27 (2H, d, *J* = 8.7 Hz, ArH), 7.54 (1H, t, *J* = 7.7 Hz, ArH), 7.72 (2H, d, *J* = 8.5 Hz, ArH), 7.79 (1H, d, *J* = 7.7 Hz, ArH), 8.07 (1H, d, *J* = 8.2 Hz, ArH), 8.22 (1H, s, ArH), 9.79 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.3 (CO aldehyde), 161.0 (CO β-lactam), 160.5, 156.2, 147.4, 135.3, 134.4, 131.4, 130.6, 129.8, 129.5, 123.4, 122.9, 118.5, 115.4, 114.6 (aromatic carbons), 80.2 (C-3 β-lactam), 56.5 (C-2 β-lactam), 55.2 (O-CH_3); GC-MS m/z = 418 [M^+]; Analysis calculated for $C_{23}H_{18}N_2O_6$: C, 66.03; H, 4.34; N, 6.70%. Found: C, 66.72; H, 4.43; N, 6.87%.

4.2.5. 4-((2-(4-Chlorophenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5e**):

White solid; Mp. 193-195 °C; IR (KBr, cm^{-1}): 1735 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 1.24 (3H, t, *J* = 6.7 Hz, CH_3), 3.93 (2H, q, *J* = 6.7 Hz, OCH_2), 5.77 (1H, d, *J* = 4.2 Hz, H-2 β-lactam), 6.01 (1H, d, *J* = 4.2 Hz, H-3 β-lactam), 6.89 (2H, d, *J* = 7.5 Hz, ArH), 7.02 (2H, d, *J* = 7.7, ArH), 7.20 (2H, d, *J* = 7.5 Hz, ArH), 7.30 (2H, d, *J* = 8.2 Hz, ArH), 7.37 (2H, d, *J* = 7.5 Hz, ArH), 7.74 (2H, d, *J* = 7.5 Hz, ArH), 9.81 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.3 (CO aldehyde), 161.1 (CO β-lactam), 160.7, 155.3, 133.0, 131.9, 131.4, 130.5, 129.9, 129.6, 128.2, 118.5, 115.4, 115.0 (aromatic carbons) , 80.1 (C-3 β-lactam), 63.2 (O-CH_2), 59.8 (C-2 β-lactam), 14.5 (CH_3); GC-MS m/z = 421 [M^+]; Analysis calculated for $C_{24}H_{20}ClNO_4$: C, 68.33; H, 4.78; N, 3.32%. Found: C, 68.27; H, 4.60; N, 3.59%.

4.2.6. 4-((1-(4-Ethoxyphenyl)-2-(4-nitrophenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5f**):

White solid; Mp. 198-200 °C; IR (KBr, cm^{-1}): 1751 (CO β-lactam), 1697 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 1.25 (3H, t, *J* = 7.0 Hz, CH_3), 3.93 (2H, q, *J* = 7.0 Hz, OCH_2) 5.93 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 6.10 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.90 (2H, d, *J* = 9.0 Hz, ArH), 7.02 (2H, d, *J* = 8.5, ArH), 7.22 (2H, d, *J* = 9.0 Hz, ArH), 7.64 (2H, d, *J* = 8.5 Hz, ArH), 7.74 (2H, d, *J* = 8.5 Hz, ArH), 8.10 (2H, d, *J* = 8.7 Hz, ArH), 9.80 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 190.5 (CO aldehyde), 161.0 (CO β-lactam), 160.8, 156.4, 148.2, 139.9, 131.7, 131.3, 129.4, 128.9, 123.7, 118.7, 115.6, 115.2 (aromatic carbons), 80.6 (C-3 β-lactam), 63.7 (O-CH_2), 60.7 (C-2 β-lactam), 14.7 (CH_3); GC-MS m/z = 432 [M^+]; Analysis calculated for $C_{24}H_{20}N_2O_6$: C, 66.66; H, 4.66; N, 6.48%. Found: C, 65.61; H, 4.45; N, 6.35%.

4.2.7. 4-((1-(4-(Dimethylamino)phenyl)-2-(4-nitrophenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (5g):

Cream solid; Mp. 160-162 °C; IR (KBr, cm^{-1}): 1743 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 2.81 (6H, s, NCH_3), 5.89 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 6.08 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.67 (2H, d, *J* = 9.0 Hz, ArH), 7.02 (2H, d, *J* = 8.5, ArH), 7.14 (2H, d, *J* = 9.0 Hz, ArH), 7.62 (2H, d, *J* = 8.5 Hz, ArH), 7.74 (2H, d, *J* = 8.5 Hz, ArH), 8.10 (2H, d, *J* = 8.5 Hz, ArH), 9.80 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.3 (CO

aldehyde), 160.7 (CO β-lactam), 160.4, 147.8, 147.4, 141.1, 131.5, 130.6, 129.4, 125.9, 123.2, 118.3, 115.4, 112.7 (aromatic carbons), 80.4 (C-3 β-lactam), 59.6 (C-2 β-lactam), 40.1 (N-CH_3); GC-MS m/z = 431 [M^+]; Analysis calculated for $C_{24}H_{21}N_3O_5$: C, 66.81; H, 4.91; N, 9.74%. Found: C, 66.51; H, 4.64; N, 9.83%.

4.2.8. 4-((2-(4-(Dimethylamino)phenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5h**):

White solid; Mp. 230-232 °C; IR (KBr, cm^{-1}): 1735 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 1.20 (3H, t, *J* = 6.7 Hz, CH_3), 2.72 (6H, s, NCH_3), 3.80 (2H, q, *J* = 6.7 Hz, OCH_2) , 5.53 (1H, d, *J* = 4.2 Hz, H-2 β-lactam), 5.84 (1H, d, *J* = 4.2 Hz, H-3 β-lactam), 6.48 (2H, d, *J* = 8.2 Hz, ArH), 6.81 (2H, d, *J* = 8.5, ArH), 6.96 (2H, d, *J* = 8.2 Hz, ArH), 7.09 (2H, d, *J* = 8.5 Hz, ArH), 7.14 (2H, d, *J* = 8.7 Hz, ArH), 7.68 (2H, d, *J* = 8.5 Hz, ArH), 9.74 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.7 (CO aldehyde), 161.6 (CO β-lactam), 159.8, 155.6, 143.1, 139.7, 131.8, 130.4, 129.4, 119.5, 118.9, 115.9, 115.4, 112.0 (aromatic carbons), 80.7 (C-3 β-lactam), 63.6 (O-CH_2), 61.1 (C-2 β-lactam), 40.5 (N-CH_3), 15.0 (CH_3); GC-MS m/z = 430 [M^+]; Analysis calculated for $C_{26}H_{26}N_2O_4$: C, 72.54; H, 6.09; N, 6.51%. Found: C, 72.28; H, 5.96; N, 6.61%.

4.2.9. 4-((2-(Anthracen-9-yl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5i**):

Cream solid; Mp. 200-202 °C; IR (KBr, cm^{-1}): 1751 (CO β-lactam), 1697 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO): 1.17 (3H, t, *J* = 7.0 Hz, CH_3), 3.81 (2H, q, *J* = 7.0 Hz, OCH_2), 6.46 (1H, d, *J* = 5.0 Hz, H-3 β-lactam), 6.74 (4H, dd, J_1= 10.2 Hz, J_2 = 2.0 Hz, ArH), 7.04 (2H, d, *J* = 8.7 Hz, ArH), 7.20 (1H,d, *J* = 5.0 Hz, H-2 β-lactam), 7.36-7.45 (4H, m, ArH), 7.54 (1H, t, *J* = 7.7 Hz, ArH), 7.70 (1H, t, *J* = 7.7 Hz, ArH), 7.97-8.07 (2H, m, ArH), 8.49 (1H, d, *J* = 9.7 Hz, ArH), 8.56 (1H, s, ArH), 8.79 (1H, d, *J* = 9.0 Hz ArH), 9.60 (1H, s, CHO); ^{13}C-NMR (100 MHz, DMSO) δ 191.0 (CO aldehyde), 161.6 (CO β-lactam), 160.7, 155.3, 131.3, 131.0, 130.9, 130.8, 130.6, 130.3, 130.2, 130.0, 129.5, 129.3, 127.3, 126.2, 125.0, 124.7, 124.4, 122.8, 121.7, 117.8, 115.0, 114.9 (aromatic carbons), 82.1 (C-3 β-lactam), 63.0 (O-CH_2) , 58.2 (C-2 β-lactam), 14.4 (CH_3); GC-MS m/z = 487 [M^+]; Analysis calculated for $C_{32}H_{25}NO_4$: C, 78.83; H, 5.17; N, 2.87%. Found: C, 76.93; H, 5.01; N, 3.02%.

4.2.10. 4-((2-(Anthracen-9-yl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)benzaldehyde (**5j**):

Cream solid; Mp. 170-172 °C; IR (KBr, cm^{-1}): 1759 (CO β-lactam), 1689 (CO aldehyde); ^{1}H-NMR (250 MHz, DMSO) δ 3.55 (3H, s, OCH_3), 6.47 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.75 (4H, d, *J* = 8.5 Hz, ArH), 7.06 (2H, d, *J* = 8.7 Hz, ArH), 7.21 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 7.35-7.44 (4H, m, ArH), 7.53 (1H, t, *J* = 7.0 Hz, ArH), 7.70 (1H, t, *J* = 8.2 Hz, ArH), 7.98 (1H, d, *J* = 8.2 Hz, ArH), 8.05 (1H, d, *J* = 8.5 Hz, ArH), 8.51 (1H, d, *J* = 8.5 Hz, ArH), 8.56 (1H, s, ArH), 8.79 (1H, d, *J* = 9.0 Hz, ArH), 9.60 (1H, s, ArH); ^{13}C-NMR (100 MHz, DMSO): 191.0 (CO aldehyde), 161.6 (CO β-lactam), 160.7, 156.0, 131.3, 130.9, 130.8, 130.6, 130.4, 130.2, 130.0, 129.5, 129.3, 128.3, 127.3, 126.2, 125.0, 124.7, 124.4, 122.8, 121.7, 117.8, 114.9, 114.6 (aromatic carbons), 82.2 (C-2 β-lactam), 58.2 (C-3 β-lactam), 55.0 (O-CH_3); GC-MS m/z = 473 [M^+]; Analysis calculated for $C_{31}H_{23}NO_4$: C, 78.63; H, 4.90; N, 2.96; O, 13.51%. Found: C, 79.03; H, 4.71; N, 3.15%.

4.3. A typical procedure for the synthesis of novel β-lactam hybrids with tetrahydro-4*H*-chromene and dihydropyrano[*c*]-chromene 8a-p:

To a mixture of *cis*-β-lactam benzaldehyde **5a-j** (1.00 mmol), malonitrile **6** (1.00 mmol) and cyclic ketone **7a,b** (1.00 mmol) in 3 mL of ethanol, DABCO (1.00 mmol) was added and the mixture was refluxed for appropriate time. After completion of the reaction, as indicated by TLC, ethanol (10 mL) was added and the reaction mixture was filtered. The crude products **8a-p** was purified by recrystallization from ethanol (95%).

4.3.1. 2-Amino-4-(4-((2-(4-chlorophenyl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8a**):

White solid; Mp. 250-252 °C; IR (KBr, cm^{-1}): 3386 (NH_2), 2191 (CN), 1743 (CO β-lactam), 1666 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.90 (3H, s, CH_3), 1.00 (3H, s, CH_3), 2.03 (1H, d, *J* = 16.0 Hz, CH_2), 2.20 (1H, d, *J* = 16.0 Hz, CH_2), 2.32-2.48 (2H, m, CH_2), 3.65 (3H, s, CH_3), 4.06 (1H, s, CH), 5.64 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 5.77 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.72 (2H, d, J = 8.2 Hz, ArH), 6.81 (2H, d, *J* = 8.0, ArH), 6.94 (4H, br, NH_2, ArH), 7.21-7.28 (4H, m, ArH), 7.38 (2H, d, *J* = 8.5 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.9 (CO ketone), 162.5, 158.9, 156.4, 155.5, 138.7, 133.2, 131.5, 130.5, 130.2, 128.5, 121.9, 120.1, 118.8, 115.5, 115.0, 113.3, 113.2 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.2 (C-3 β-lactam), 60.5 (C-2 β-lactam), 58.5 (C-CN), 55.6 (O-CH_3), 50.3 (CH_2), 35.0 (CH_2), 32.1 (CH), 28.8 (CH_3), 27.0 (CH_3); GC-MS m/z = 595 [M^+]; Analysis calculated for $C_{34}H_{30}ClN_3O_5$: C, 68.51; H, 5.07; N, 7.05%. Found: C, 63.64; H, 4.69; N, 6.90%.

4.3.2. 2-Amino-4-(4-((2-(4-chlorophenyl)-4-oxo-1-(p-tolyl)azetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8b**):

White solid; Mp. 208-210 °C; IR (KBr, cm^{-1}): 3386, 3325 (NH_2), 2198 (CN), 1743 (CO β-lactam), 1666 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.88 (3H, s, CH_3), 1.00 (3H, s, CH_3), 2.04 (1H, d, *J* = 16.2 Hz, CH_2), 2.18-2.25 (4H, m, CH_3 and CH_2), 2.38-2.47 (2H, m, CH_2), 4.07 (1H, s, CH), 5.69 (1H, d, *J* = 5.0 Hz, H-2 β-lactam), 5.82 (1H, d, *J* = 5.0 Hz, H-3 β-lactam), 6.72 (2H, d, *J* = 7.0, ArH), 6.88-6.96 (4H, m, ArH, NH_2), 7.10-7.18 (4H, m, ArH), 7.27-7.34 (4H, m, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.9 (CO ketone), 162.9, 162.5, 158.9, 155.5, 139.7, 138.7,134.4, 134.0, 133.3, 132.7, 130.2, 128.5, 120.1, 118.8, 117.4, 115.5, 113.3 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.2 (C-3 β-lactam), 60.5 (C-2 β-lactam), 58.5 (C-CN), 50.3 (CH_2), 35.1 (CH_2), 32.1 (CH), 28.8 (CH_3), 27.0 (CH_3), 20.8 (CH_3); GC-MS m/z = 579 [M^+]; Analysis calculated for $C_{34}H_{30}ClN_3O_4$: C, 70.40; H, 5.21; N, 7.24%. Found: C, 69.93; H, 5.11; N, 7.61 %.

4.3.3. 2-Amino-4-(4-((1-(4-chlorophenyl)-2-(3-nitrophenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8c**):

White solid; Mp. 219-221 °C; IR (KBr, cm^{-1}): 3417, 3332 (NH_2), 2191 (CN), 1759 (CO β-lactam), 1674 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.87 (3H, s, CH_3), 1.00 (3H, s, CH_3), 2.03 (1H, d, *J* = 16.0 Hz, CH_2), 2.20 (1H, d, *J* = 16.0 Hz, CH_2), 2.36-2.48 (2H, m, CH_2), 4.04 (1H, s, CH), 5.93 (2H, br, H-2 and H-3 β-lactam), 6.71 (2H, d, *J* = 8.2 Hz, ArH), 6.91-6.94 (4H, m, ArH, NH_2), 7.32 (2H, d, *J* = 8.2 Hz, ArH), 7.40 (2H, d, *J* = 8.2 Hz, ArH), 7.54 (1H, t, *J* = 7.7

Hz, ArH), 7.77 (1H, d, *J* = 6.2 Hz, ArH), 8.07 (1H, d, *J* = 8.0 Hz, ArH), 8.22 (1H, d, *J* = 8.0 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.4 (CO ketone), 167.7 (CO β-lactam), 166.8, 163.7, 158.4, 157.3, 149.2, 147.9, 138.4, 137.1, 129.8, 129.4, 128.8, 128.3, 128.1, 126.6, 124.9, 123.4, 119.8, 118.7 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.0 (C-3 β-lactam), 64.9 (C-2 β-lactam), 59.8 (C-CN), 49.8 (CH_2), 34.6 (CH_2), 31.7 (CH), 28.3 (CH_3), 26.6 (CH_3); GC-MS m/z = 610 [M^+]; Analysis calculated for $C_{33}H_{27}ClN_4O_6$: C, 64.87; H, 4.45; N, 9.17%. Found: C, 64.63; H, 4.31; N, 9.61%.

4.3.4. 2-Amino-4-(4-((1-(4-methoxyphenyl)-2-(3-nitrophenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8d**):

Cream solid; Mp. 228-230 °C; IR (KBr, cm^{-1}): 3448, 3332 (NH_2), 2191 (CN), 1759 (CO β-lactam), 1666 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.87 (3H, s, CH_3), 1.00 (3H, s, CH_3), 2.03 (1H, d, *J* = 16.0 Hz, CH_2), 2.20 (1H, d, *J* = 16.0 Hz, CH_2), 2.36-2.48 (2H, m, CH_2), 3.67 (3H, s, CH_3), 4.04 (1H, s, CH), 5.88 (2H, br, H-2 and H-3 β-lactam), 6.70 (2H, d, *J* = 8.2 Hz, ArH), 6.91-6.94 (6H, m, ArH, NH_2), 7.24 (2H, d, *J* = 8.2 Hz, ArH), 7.53 (1H, t, *J* = 8.0 Hz, ArH), 7.77 (1H, d, *J* = 7.2 Hz, ArH), 8.05 (1H, d, *J* = 7.7 Hz, ArH), 8.19 (1H, s, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.4 (CO ketone), 168.7, 161.3, 158.1, 155.0, 152.7, 144.1, 135.4, 134.1, 128.8, 128.2, 127.3, 126.0, 124.7, 123.0, 118.3, 116.0, 115.0, 113.9, 112.4 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 80.9 (C-3 β-lactam), 60.4 (C-2 β-lactam), 59.1 (C-CN), 54.1 (O-CH_3) , 50.3 (CH_2), 34.6 (CH_2), 31.7 (CH), 28.5 (CH_3), 27.1 (CH_3); GC-MS m/z = 606 [M^+]; Analysis calculated for $C_{34}H_{30}N_4O_7$: C, 67.32; H, 4.98; N, 9.24%. Found: C, 66.93; H, 4.71; N, 9.61%.

4.3.5. 2-Amino-4-(4-((2-(4-chlorophenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8e**):

White solid; Mp. 242-244 °C; IR (KBr, cm^{-1}): 3394 (NH_2), 2191 (CN), 1735 (CO β-lactam), 1666 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.88 (3H, s, CH_3), 1.00 (3H, s, CH_3), 1.25 (3H, t, *J* = 6.7 Hz, CH_3), 2.03 (1H, d, *J* = 16.2 Hz, CH_2), 2.21 (1H, d, *J* = 16.2 Hz, CH_2), 2.37-2.47 (2H, m, CH_2) 3.92 (2H, q, *J* = 6.7 Hz, OCH_2), 4.06 (1H, s, CH), 5.66 (1H, d, *J* = 4.0 Hz, H-2 β-lactam), 5.79 (1H, d, *J* = 4.0 Hz, H-3 β-lactam), 6.71 (2H, d, *J* = 7.5 Hz, ArH), 6.86-6.95 (6H, m, ArH, NH_2), 7.18 (2H, d, *J* = 7.5 Hz, ArH), 7.27-7.34 (4H, m, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.9 (CO ketone), 162.5, 158.4, 155.6, 155.5, 147.1, 139.7, 138.7, 133.3, 132.8, 130.2, 130.1, 128.6, 128.5, 120.0, 118.8, 115.5, 113.3 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.2 (C-3 β-lactam), 63.6 (O-CH_2), 60.5 (C-2 β-lactam), 58.5 (C-CN), 50.3 (CH_2) , 35.0 (CH_2), 32.1 (CH), 28.8 (CH_3), 27.0 (CH_3), 14.9 (CH_3); GC-MS m/z = 609 [M^+]; Analysis calculated for $C_{35}H_{32}ClN_3O_5$: C, 68.90; H, 5.29; N, 6.89%. Found: C, 66.47; H, 4.83; N, 6.73%.

4.3.6. 2-Amino-4-(4-((1-(4-ethoxyphenyl)-2-(4-nitrophenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8f**):

Cream solid; Mp. 237-239 °C; IR (KBr, cm^{-1}): 3448, 3386, 3317 (NH_2), 2191 (CN), 1751 (CO β-lactam), 1666 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.86 (3H, s, CH_3), 0.99 (3H, s, CH_3), 1.24 (3H, t, *J* = 6.5 Hz, CH_3), 2.02 (1H, d, *J* = 16.0 Hz, CH_2), 2.20 (1H, d, *J* = 16.0 Hz, CH_2), 2.36-2.47 (2H, m, CH_2), 3.93 (2H, q, *J* = 16.0 Hz, OCH_2), 4.05 (1H, s, CH), 5.83 (1H, d, *J* = 4.0

Hz, H-2 β-lactam), 5.89 (1H, d, *J* = 4.0 Hz, H-3 β-lactam), 6.71 (2H, d, *J* = 8.0 Hz, ArH), 6.89-6.94 (6H, m, ArH, NH_2), 7.19 (2H, d, *J* = 8.2 Hz, ArH), 7.59 (2H, d, *J* = 6.7 Hz, ArH), 8.08 (2H, d, *J* = 7.2 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.5 (CO ketone), 167.8, 161.8, 158.3, 154.9, 147.4, 141.2, 139.4, 138.5, 129.6, 129.3, 128.2, 123.5, 123.3, 121.6, 119.6, 118.3, 115.1 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.1 (C-3 β-lactam), 63.2 (O-CH_2), 60.0 (C-2 β-lactam), 58.0 (C-CN), 49.8 (CH_2), 34.7 (CH_2), 31.7 (CH), 28.4 (CH_3), 26.5 (CH_3), 14.5 (CH_3); GC-MS m/z = 620 [M^+]; Analysis calculated for $C_{35}H_{32}N_4O_7$: C, 67.73; H, 5.20; N, 9.03%. Found: C, 66.40; H, 5.27; N, 9.22%.

4.3.7. 2-Amino-4-(4-((1-(4-(dimethylamino)phenyl)-2-(4-nitrophenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8g**):

Cream solid; Mp. 288-290 °C; IR (KBr, cm^{-1}): 3456, 3325 (NH_2), 2198 (CN), 1743 (CO β-lactam), 1674 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.86 (3H, s, CH_3), 0.99 (3H, s, CH_3), 2.02 (1H, d, *J* = 16.0 Hz, CH_2), 2.21 (1H, d, *J* = 16.0 Hz, CH_2), 2. 36-2.48 (2H, m, CH_2), 2.80 (6H, s, NCH_3), 4.05 (1H, s, CH), 5.79 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 5.87 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.66 (2H, d, *J* = 9.0 Hz, ArH), 6.71 (2H, d, *J* = 8.5 Hz, ArH), 6.91-6.94 (4H, m, ArH, NH_2), 7.12 (2H, d, *J* = 9.0 Hz, ArH), 7.57 (2H, d, *J* = 8.7 Hz, ArH) 8.08 (2H, d, *J* = 8.5 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.9 (CO ketone), 162.6, 161.7, 158.8, 155.4, 149.9, 148.1, 142.0, 139.7, 138.8, 129.7, 128.5, 126.6, 123.6, 120.1, 118.7, 115.5, 113.2 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.4 (C-3 β-lactam), 60.3 (C-2 β-lactam), 58.6 (C-CN), 50.3 (CH_2), 40.5 (N-CH_3), 35.1 (CH_2), 32.1 (CH), 28.8 (CH_3), 27.0 (CH_3); GC-MS m/z = 619 [M^+]; Analysis calculated for $C_{35}H_{33}N_5O_6$: C, 67.84; H, 5.37; N, 11.30%. Found: C, 66.93; H, 5.31; N, 12.01%.

4.3.8. 2-Amino-4-(4-((-2-(4-(dimethylamino)phenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8h**):

Cream solid; Mp. 253-255 °C; IR (KBr, cm^{-1}): 3394, 3325 (NH_2), 2191 (CN), 1743 (CO β-lactam), 1681 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.89 (3H, s, CH_3), 0.99(3H, s, CH_3), 1.25 (3H, t, *J* = 7.0 Hz, CH_3) 2.04 (1H, d, *J* = 16.2 Hz, CH_2), 2.20 (1H, d, *J* = 16.2 Hz, CH_2), 2.38-2.48 (2H, m, CH_2), 2.80 (6H, s, NCH_3), 3.92 (2H, q, *J* = 7.0 Hz, OCH_2), 4.06 (1H, s, CH), 5.48 (1H, d, *J* = 4.5 Hz, H-2 β-lactam), 5.67 (1H, d, *J* = 4.5 Hz, H-3 β-lactam), 6.56 (2H, d, *J* = 8.5 Hz, ArH), 6.72 (2H, d, *J* = 8.2 Hz, ArH), 6.85 (2H, d, *J* = 8.7 Hz, ArH), 6.92-6.95 (4H, m, ArH, NH_2); 7.12 (2H, d, *J* = 8.5 Hz, ArH), 7.19 (2H, d, *J* = 8.7 Hz, ArH); ^{13}C-NMR (100MHz, DMSO) δ 196.0 (CO ketone), 162.7, 155.9, 155.2, 150.3, 147.6, 138.4, 135.3, 130.4, 129.2, 128.3, 123.6, 122.6, 118.7, 115.6, 115.2, 113.1, 111.9 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.2 (C-3 β-lactam), 63.3 (O-CH_2), 61.1 (C-2 β-lactam), 58.4 (C-CN), 50.2 (CH_2), 40.3 (N-CH_3), 34.8 (CH_2), 31.9 (CH), 28.5 (CH_3), 26.8 (CH_3), 14.7 (CH_3); GC-MS m/z = 618 [M^+] Analysis calculated for $C_{37}H_{38}N_4O_5$: C, 71.83; H, 6.19; N, 9.06%. Found: C, 69.93; H, 6.01; N, 9.61%.

4.3.9. 2-Amino-4-(4-((2-(anthracen-9-yl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8i**):

White solid; Mp. 220-222 °C; IR (KBr, cm^{-1}): 3456, 3332 (NH_2), 2191 (CN), 1751 (CO β-lactam), 1674 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.83 (3H, s, CH_3), 0.96 (3H, s, CH_3),

1.17 (3H, t, *J* = 7.2 Hz, CH_3) 1.98 (1H, d, *J* = 16.2 Hz, CH_2), 2.14 (1H, d, *J* = 16.2 Hz, CH_2), 2.33-2.40 (2H, m, CH_2), 3.81 (2H, q, *J* = 7.2 Hz, OCH_2), 3.94 (1H, s, CH), 6.20 (1H, d, *J* = 5.0 Hz, H-3 β-lactam), 6.54 (2H, d, *J* = 8.5 Hz, ArH), 6.71-6.77 (4H, m, ArH), 6.89 (2H, s, NH_2), 7.02 (2H, d, *J* = 8.7 Hz, ArH), 7.11 (1H, d, *J* = 5.0 Hz, H-2 β-lactam), 7.36-7.40 (2H, m, ArH), 7.56 (1H, t, *J* = 6.7 Hz, ArH), 7.68 (1H, t, *J* = 8.5 Hz, ArH), 7.99-8.02 (1H, m, ArH), 8.12 (1H, d, m, ArH), 8.45-8.49 (1H, m, ArH), 8.61 (1H, s, ArH), 8.72 (1H, d, *J* = 9.0 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.4 (CO ketone), 162.7, 162.1, 158.2, 155.5, 155.2, 131.2, 131.0, 130.7, 130.4, 129.8, 129.6, 129.3, 127.9, 127.8, 127.2, 127.1, 126.0, 125.0, 124.7, 124.6, 122.7, 122.4, 119.5, 117.8, 115.1, 115.0, 112.5 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 83.1 (C-2 β-lactam), 63.0 (O-CH_2), 58.6 (C-3 β-lactam), 58.2 (C-CN), 49.8 (CH_2), 34.5 (CH_2), 31.6 (CH), 28.3 (CH_3), 26.8 (CH_3), 14.4 (CH_3); GC-MS m/z = 675 [M^+] Analysis calculated for $C_{43}H_{37}N_3O_5$: C, 76.43; H, 5.52; N, 6.22%. Found: C, 76.03; H, 5.31; N, 6.61%.

4.3.10. 2-Amino-4-(4-((2-(anthracen-9-yl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (**8j**):

White solid; Mp. 202-204 °C; IR (KBr, cm^{-1}): 3448, 3325 (NH_2), 2191 (CN), 1748 (CO β-lactam), 1674 (CO ketone); ^{1}H-NMR (250 MHz, DMSO): 0.83 (3H, s, CH_3), 0.96 (3H, s, CH_3), 1.98 (1H, d, *J* = 16.0 Hz, CH_2), 2.14 (1H, d, *J* = 16.0 Hz, CH_2), 2.31-2.40 (2H, m, CH_2), 3.56 (3H, s, CH_3), 3.94 (1H, s, CH), 6.21 (1H, d, *J* = 5.0 Hz, H-3 β-lactam), 6.54 (2H, d, *J* = 8.5 Hz, ArH), 6.73-6.77 (4H, m, ArH), 6.89 (2H, s, NH_2), 7.03 (2H, d, *J* = 9.0 Hz, ArH), 7.12(1H, d, *J* = 5.0 Hz, H-2 β-lactam); 7.36-7.40 (2H, m, ArH), 7.56-7.59 (1H, m, ArH), 7.66-7.72 (1H, m, ArH), 7.99-8.03 (1H, m, ArH), 8.11 (1H, t, *J* = 7.4 Hz, ArH), 8.45-8.49 (1H, m, ArH), 8.61 (1H, s, ArH), 8-68-8.74 (1H, m, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 195.4 (CO ketone), 162.7, 162.0, 158.3, 155.9, 155.5, 138.3, 131.2, 131.0, 130.7, 130.5, 129.7, 129.6, 129.5, 129.3, 127.9, 127.8, 127.1, 126.0, 125.0, 124.7, 124.6, 122.8, 122.4, 119.5, 117.8, 115.1, 114.5 (CO β-lactam, vinylic carbon, aromatic carbons and CN), 83.0 (C-2 β-lactam), 58.6 (C-3 β-lactam), 58.0 (C-CN), 55.0 (O-CH_3), 49.8 (CH_2), 34.5 (CH_2), 31.6 (CH), 28.3 (CH_3), 26.5 (CH_3); GC-MS m/z = 661 [M^+]; Analysis calculated for $C_{42}H_{35}N_3O_5$: C, 76.23; H, 5.33; N, 6.35%. Found: C, 65.93; H, 5.07; N, 6.82%.

4.3.11. 2-Amino-4-(4-((2-(4-chlorophenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-*c*]chromene-3-carbonitrile (**8k**):

White solid; Mp. 210-212 °C; IR (KBr, cm^{-1}): 3448 , 3325 (NH_2), 2198 (CN), 1759 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 1.25 (3H, t, *J* = 7.0 Hz, CH_3) 3.92 (2H, q, *J* = 7.0 Hz, OCH_2), 4.34 (1H, s, CH), 5.66 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 5.81 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.75 (2H, d, *J* = 8.5 Hz, ArH), 6.87 (2H, d, *J* = 8.7 Hz, ArH), 7.07 (2H, d, *J* = 8.5 Hz, ArH), 7.18 (2H, d, *J* = 8.7 Hz, ArH); 7.28-7.34 (6H, m, ArH, NH_2), 7.41-7.49 (2H, m, ArH), 7.69 (1H, t, *J* = 7.7 Hz, ArH), 7.86 (1H, d, *J* = 7.7 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 162.0, 159.4, 157.8, 155.6, 155.2, 153.2, 152.0, 136.9, 132.9, 132.3, 129.8, 129.7, 128.6, 128.2 , 124.6, 122.4, 119.1, 118.4, 116.8, 116.5, 115.3, 115.0, 112.9, 103.9 (CO lactone, CO β-lactam, vinylic carbon, aromatic carbons and CN), 80.8 (C-3 β-lactam), 63.1 (O-CH_2), 60.0 (C-2 β-lactam), 57.9 (C-CN), 36.0 (CH), 14.5 (CH_3); GC-MS m/z = 631 [M^+]; Analysis calculated for $C_{36}H_{26}ClN_3O_6$: C, 68.41; H, 4.15; Cl, 5.61; N, 6.65%. Found: C, 68.03; H, 4.09; N, 7.11%.

4.3.12. 2-Amino-4-(4-((2-(4-chlorophenyl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-*c*]chromene-3-carbonitrile (**8l**):

White solid; Mp. 199-201 °C; IR (KBr, cm^{-1}): 3463, 3325 (NH_2), 2198 (CN), 1743 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 3.67 (3H, s, CH_3), 4.35 (1H, s, CH), 5.67 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 5.82 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.75-6.79 (2H, d, *J* = 8.5 Hz, ArH), 6.89 (2H, d, *J* = 8.7 Hz, ArH), 7.07 (2H, d, *J* = 8.0 Hz, ArH), 7.20 (2H, d, *J* = 8.7 Hz, ArH), 7.28-7.35 (6H, m, ArH, NH_2), 7.41-7.49 (2H, m, ArH), 7.69 (1H, t, *J* = 7.7 Hz, ArH), 7.86 (1H, d, *J* = 7.7 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 162.0, 161.9, 159.4, 157.9, 157.8, 155.9, 155.5, 153.2, 153.0, 152.0, 132.8, 132.3, 129.8, 128.6, 128.1, 124.6, 122.4, 119.1, 118.4, 116.5, 115.2, 114.5, 112.9, 103.9 (CO lactone, CO β-lactam, vinylic carbon, aromatic carbons and CN), 80.8 (C-3 β-lactam), 60.0 (C-2 β-lactam), 57.8 (C-CN), 55.2 (O-CH_3), 36.0 (CH); GC-MS m/z = 661 [M^+]; Analysis calculated for $C_{35}H_{24}ClN_3O_6$: C, 68.02; H, 3.91; Cl, 5.74; N, 6.80; O, 15.53%. Found: C, 66.93; H, 3.21; N, 5.96%.

4.3.13. 2-Amino-4-(4-((1-(4-(dimethylamino)phenyl)-2-(4-nitrophenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-*c*]chromene-3-carbonitrile (**8m**):

White solid; Mp. 222-224 °C; IR (KBr, cm^{-1}): 3325 (NH_2), 2198 (CN), 1735 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 2.79 (6H, s, NCH_3), 4.33 (1H, s, CH), 5.79 (1H, d, *J* = 4.5 Hz, H-2 β-lactam), 5.89 (1H, d, *J* = 4.5 Hz, H-3 β-lactam), 6.65 (2H, d, *J* = 9.0 Hz, ArH), 6.74 (2H, d, *J* = 8.5 Hz, ArH), 7.04-7.13 (4H, m, ArH), 7.34 (2H, s, NH_2), 7.40-7.48 (2H, m, ArH), 7.56 (2H, d, *J* = 8.7 Hz, ArH), 7.68 (1H, t, *J* = 7.7 Hz, ArH), 7.85 (1H, d, *J* = 7.7 Hz, ArH), 8.07 (2H, d, *J* = 8.5 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 161.3, 159.4, 157.8, 155.4, 153.1, 152.0, 147.7, 147.3, 141.5, 137.0, 132.8, 129.3, 128.7, 126.1, 124.6, 123.1, 122.4, 119.1, 118.2, 116.5, 115.2, 112.9, 112.7, 103.9 (CO lactone, CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.0 (C-3 β-lactam), 59.8 (C-2 β-lactam), 57.8 (C-CN), 40.1 (N-CH_3), 36.1 (CH); GC-MS m/z = 641 [M^+] Analysis calculated for $C_{36}H_{27}N_5O_7$: C, 67.39; H, 4.24; N, 10.91%. Found: C, 66.93; H, 3.98; N, 4.61%.

4.3.14. 2-Amino-4-(4-((2-(4-(dimethylamino)phenyl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-c]chromene-3-carbonitrile (**8n**):

Cream solid; Mp. 197-199 °C; IR (KBr, cm^{-1}): 3325 (NH_2), 2198 (CN), 1759 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 1.25 (3H, t, *J* = 7.0 Hz, CH_3), 2.78 (3H, s, NCH_3), 2.80 (3H, s, NCH_3), 3.91 (2H, q, *J* = 7.0 Hz, OCH_2), 4.34 (1H, s, CH), 5.48 (1H, d, *J* = 4.7 Hz, H-2 β-lactam), 5.69 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.56 (2H, d, *J* = 7.2 Hz, ArH), 6.75 (2H, d, *J* = 8.2 Hz, ArH), 6.85 (2H, d, *J* = 8.7 Hz, ArH), 7.05-7.11 (3H, m, ArH), 7.17 (3H, t, *J* = 8.7 Hz, ArH), 7.33 (2H, s, NH_2), 7.42 (1H, d, *J* = 7.5 Hz, ArH), 7.47 (1H, d, *J* = 7.5 Hz, ArH), 7.68 (1H, t, *J* = 7.5 Hz, ArH), 7.85 (1H, d, *J* = 7.7 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 162.3, 159.4, 157.9, 156.0, 155.0, 153.1, 152.0, 150.0, 136.6, 132.8, 130.1, 128.8, 128.5, 124.5, 122.4, 119.5, 119.1, 118.4, 116.5, 115.4, 114.9, 112.9, 111.7, 104.0 (CO lactone, CO β-lactam, vinylic carbon, aromatic carbons and CN), 81.0 (C-3 β-lactam), 63.1 (O-CH_2), 60.9 (C- 2β-lactam), 57.3 (C-CN), 40.1 (N-CH_3), 36.0 (CH), 14.5 (CH_3); GC-MS m/z = 640 [M^+]; Analysis calculated for $C_{38}H_{32}N_4O_6$: C, 71.24; H, 5.03; N, 8.74%. Found: C, 70.93; H, 4.57; N, 8.95 %.

4.3.15. 2-Amino-4-(4-((2-(anthracen-9-yl)-1-(4-ethoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-c]chromene-3-carbonitrile (**8o**):

Cream solid; Mp. 226-228 °C; IR (KBr, cm^{-1}): 3402, 3325 (NH_2), 2198 (CN), 1743 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 1.17 (3H, t, *J* = 7.0 Hz, CH_3), 3.81 (2H, q, *J* = 7.0 Hz, OCH_2), 4.20 (1H, s, CH), 6.23 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.51 (2H, d, *J* = 8.2 Hz, H-2 β-lactam), 6.73 (2H, d, *J* = 8.7 Hz, ArH), 6.83 (2H, d, *J* = 8.2 Hz, ArH), 7.03 (2H, d, J = 8.7 Hz, ArH), 7.09 (1H, d, *J* = 4.7 Hz, H-2 β-lactam); 7.29 (2H, s, NH_2), 7.33-7.39 (3H, m, ArH), 7.46 (2H, t, *J* = 8.0 Hz, ArH), 7.60 (1H, t, *J* = 8.2 Hz, ArH), 7.69 (1H, t, *J* = 8.0 Hz, ArH), 7.82 (1H, d, *J* = 7.7 Hz, ArH), 7.97 (2H, d, *J* = 8.2 Hz, ArH), 8.45-8.49 (1H, m, ArH), 8.54 (1H, s, ArH), 8.66 (1H, d, *J* = 9.0 Hz, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 162.6, 159.2, 157.9, 157.3, 156.4, 154.4, 136.6, 131.0, 130.6, 130.4, 129.7, 129.5, 129.3, 128.8, 128.3, 127.0, 126.0, 124.6, 123.9, 122.7, 122.3, 121.3, 120.3, 119.7, 119.1, 118.7, 117.8, 116.5, 115.1, 115.0, 114.4, 113.2, 112.9, 104.0 (CO β-lactam, CO lactone, vinylic carbon, aromatic carbons and CN), 82.8 (C-2 β-lactam), 63.0 (O-CH_2), 61.7 (C-3 β-lactam), 59.6 (C-CN), 35.9 (CH), 14.4 (CH_3); GC-MS m/z = 697 [M^+]; Analysis calculated for $C_{44}H_{31}N_3O_6$: C, 75.74; H, 4.48; N, 6.02%. Found: C, 74.93; H, 4.13; N, 6.61%.

4.3.16. 2-Amino-4-(4-((2-(anthracen-9-yl)-1-(4-methoxyphenyl)-4-oxoazetidin-3-yl)oxy)phenyl)-5-oxo-4*H*,5*H*-pyrano[3,2-*c*]chromene-3-carbonitrile (**8p**):

Cream solid; Mp. 212-214 °C; IR (KBr, cm^{-1}): 3425, 3325 (NH_2), 2198 (CN), 1743 (CO β-lactam), 1674 (CO lactone); ^{1}H-NMR (250 MHz, DMSO): 3.56 (3H, s, OCH_2), 4.20 (1H, s, CH), 6.23 (1H, d, *J* = 4.7 Hz, H-3 β-lactam), 6.52 (2H, d, *J* = 8.0 Hz, ArH), 6.75 (2H, d, *J* = 9.0 Hz, ArH), 6.83 (2H, d, *J* = 8.5 Hz, ArH), 7.02-7.09 (3H, m, ArH, H-2 β-lactam), 7.30-7.46 (7H, m, ArH); 7.53-7.69 (2H, m, ArH), 7.77-7.84 (1H, m, ArH), 7.96-8.11 (2H, m, ArH), 8.45-8.68 (3H, m, ArH); ^{13}C-NMR (100 MHz, DMSO) δ 162.6, 159.2, 157.9, 157.8, 155.9, 155.7, 153.1, 152.8, 152.0, 136.6, 132.8, 131.2, 131.0, 130.6, 130.5, 129.7, 129.3, 128.3, 127.0, 126.0, 125.0, 124.7, 124.5, 122.7, 122.3, 122.2, 119.0, 117.8, 116.5, 115.3, 115.1, 114.5, 112.9, 104.0 (CO β-lactam, CO lactone, vinylic carbon, aromatic carbons and CN), 82.8 (C-2 β-lactam), 58.4 (C-3 β-lactam), 57.6 (C-CN), 55.0 (O-CH_3), 35.9 (CH); GC-MS m/z = 683 [M^+]; Analysis calculated for $C_{43}H_{29}N_3O_6$: C, 75.54; H, 4.28; N, 6.15%. Found: C, 74.93; H, 4.01; N, 6.91%.

4.4. *In vitro* antiinflammatory activity

4.4.1. Principle of the assay

The *in vitro* anti-inflammatory assay is based on the ability of macrophages to generate a strong inflammatory response when stimulated with antigens. Mouse immortalized macrophages (RAW 264.7 cell line) are stimulated by *E. coli* LPS, and exposed to the test material for 24 hours. At the end of the incubation period, NO production is evaluated indirectly by measuring the accumulation of nitrite/nitrate, the stable end-products of NO oxidation, in the culture medium using a spectrophotometric method based on the Griess reaction.

4.4.2. Cell line

Mouse macrophages (RAW 264.7, Sigma-Aldrich, N° P6110401, Lot. 09I006), low passage number (<50).

4.4.3. Culture medium

Complete medium: DMEM with stable L-glutamine (Dulbecco's Minimum Essential Medium, PAN BIOTECH. Lot 974251) supplemented with penicillin 100 IU/ml and streptomycin 100 µg/mL (PAN BIOTECH, Lot 20145123), and 10% of inactivated calf serum (PAN BIOTECH, Lot P440008), pH 7.2, freshly prepared, stored no longer than 3 weeks.

4.4.4. Dilutions of the test material

The test materials were diluted into dimethyl sulfoxide (DMSO, Sigma-Aldrich).

4.4.5. Controls

Negative control: DMSO (Sigma-Aldrich).
Positive control: Dexamethasone (Sigma-Aldrich) 1 – 5 – 10 – 50 – 100 µM.

4.4.6. Test procedure

Cells were seeded into 48-well tissue culture plates at the concentration of 1.10^5 cells/mL (200 µL/well) for 24 hours at 37°C (5% CO_2). At the end of the incubation period the culture medium was replaced by 200 µL of medium containing the appropriate concentrations of the test materials, and cells were incubated at 37°C (5% CO_2) during one hour. At the end of the incubation period, pro-inflammatory LPS from *E. coli* was added to cell cultures (1µg/mL). Then cells were incubated at 37°C (5% CO_2) during 24 hours.

4.4.7. Assessment of NO release

NO release was measured in the culture supernatant by the Griess reaction. 100 µL of the supernatants were transferred into the wells of a 96-well tissue culture plate, and 100 µL of the Griess modified reagent (SIGMA-ALDRICH) were added in each well. After a 15 min period at room temperature, the Optical Density (OD) of each well was read at 540 nm by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The results obtained for wells treated with the test material were compared to those of untreated control wells (DMSO, 100% viability) and converted to percentage values.

4.4.8. Assessment of cell viability

In parallel to the assessment of NO release, cell viability was measured to validate the assay. The WST-1 vital dye reagent was used to measure cell mitochondrial respiration. For this purpose, the culture medium was decanted and 100µl of WST-1 reagent (1/10 dilution) were added in each well. After a 30-min incubation period at 37°C (5% CO2), the Optical Density (OD) of each well was read at 450 nm by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The results obtained for wells treated with the test material were compared to those of untreated control wells (DMSO, 100% viability) and converted to percentage values [26].

4.4.9. Calculation of the IC_{50}

Inhibition of NO release and inhibition of cell viability were expressed as percentages as compared to the negative controls:

$$\text{Percentage of NO release} = \frac{100 \times (\text{OD of test well} - \text{OD of blank})}{\text{OD of DMSO control} - \text{OD of blank}}$$

$$\text{Percentage of Cell viability} = \frac{100 \times (\text{OD of test well} - \text{OD of blank})}{\text{OD of DMSO control} - \text{OD of blank}}$$

The concentrations of the test material causing respectively a 50% decrease of NO release ($IC_{50\text{-NO release}}$) and a 50% decrease of cell viability ($IC_{50\text{-cell viability}}$) were calculated using software Tablecurve Version 2.0. The anti-inflammatory ratio corresponded to the ratio between the anti-inflammatory activity and the toxicity. It was expressed as follows:

$$\text{Anti-inflammatory ratio} = IC_{50\text{-cell viability}} / IC_{50\text{-NO release}}$$

4.5. Cytotoxicity and Anticancer study

The cytotoxicity assay was performed as described by Rowan et al. (2001) with modifications. The *HepG2* and *SW1116* cell line were used in which the cell monolayers were seeded at 5×10^4 cells per well (in RPMI 1640 medium plus 10% fetal calf serum) in 96-well plate. Various concentration of tested compounds (5,10, 50, 100 and 200 μM) were prepared and inoculated to cell monolayer and repeated triple for each compound. Monolayers containing the tested compounds were incubated overnight at 37 °C in a 5% CO2 atmosphere. After overnight incubation, the suspension from each well was discarded and 25 μl of fresh complete medium (RPMI+10% fetal calf serum) containing 0.004 g/mL of MTT reagent (Sigma, Ronkonkoma, NY, USA) was added. Samples were incubated for 3 hours at 37 °C in 5% CO_2 and the formazan product was solubilized by the addition of 100 ml of dimethyl sulfoxide. Optical densities of the suspensions were measured at 540 nm using an ELISA reader (Biotek, Power Wave, Winooski, VT, USA) and cytotoxicity (percentage of dead cells) calculated as (1 – optical density of test sample/optical density of negative control)×100 [27].

4.6. Molecular docking study

Computer-simulated docking studies were accomplished by the AutoDock 4.2 software [28]. Lamarckian Genetic Algorithm of the AutoDock 4.2 program was used as the search algorithm. The Graphical User Interface program AutoDock Tools 1.5.6 (ADT) were used to prepare, run, and analyze the docking simulations.[29] Molecular docking of compounds was performed with two crystal structures (PDB ID: 4NOS (human inducible nitric oxide synthase), 3LN1 (cyclooxygenase-2)) by the Auto-Dock Tool 1.5.6. All two-dimensional (2D) structures of the compounds were built using the ChemDraw program (ChemDraw Ultra 10.0, Cambridge soft.), and then moved into the Hyperchem 8.0 software (HyperChem, Release 8.0 for Windows, Molecular Modeling System: HyperCube, 2007). Molecules were subjected to energy minimization with MM+ force field and then PM3 semi-empirical technique. Then the partial

charges of atoms were calculated by the Gasteiger–Marsili procedure implemented in the AutoDock Tools package [29]. The non-polar hydrogens of compounds were merged. The crystal structures of protein were taken from Protein Data bank (www.rcsb.org). All bound water and ligands were eliminated from the protein, and polar hydrogen atom were added to the protein as it was required for the electrostatics interactions, and then non-polar hydrogen atoms were merged together. In all dockings, a grid map with 60 grid points in the X, Y, and Z directions was built. Among the three different search algorithms offered by AutoDock 4.2, the Lamarckian genetic algorithm (LGA) approach was applied. For all docking procedures, 150 independent runs with the step sizes of 0.2 Å for translations and 5° for orientations and torsions were considered. For the Lamarckian GA method, a maximum number of 25×10^5 energy evaluations; 27,000 maximum generations; a gene mutation rate of 0.02; and a cross-over rate of 0.8 were used. At the end of docking, the structures were ranked by energy. Ligand-receptor interactions were all visualized on the basis of docking results using Discovery Studio Visualizer 4.0 and Ligplus2012.

Conflict of interest

The authors declare that they have no conflict of interest.

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Notes

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ABBREVIATIONS

MCRs, Multicomponent Reactions; ORTEP, Oak Ridge Thermal Ellipsoid Plot; DABCO, 1,4-Diazabicyclo[2.2.2]octane; NSAIDs, Non-steroidal Anti-inflammatory Drugs; NO, Nitric Oxide;

IC50, Half Maximal Inhibitory Concentration; MTT, Methyl Thiazol Tetrazolium bromide; MTX, Methotrexate; RMSD, Root-Mean-Square Deviation; PDB ID, Protein Data Bank ID; CLogP, Calculation LogP; OD, Optical Density;

Appendix A. Supplementary data

Electronic Supplementary Information (ESI) available: Spectra for new compounds (IR, ^{1}H NMR, ^{13}C NMR spectral data), elemental analysis, molecular docking studies data, crystallographic data and structure determination and General experimental methods**.**

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- ✓ Some novel chromeno β-lactam hybrids have been synthesized.
- ✓ Inflammatory, mammalian cell toxicity and cancer activities have been examined.
- ✓ **5b** and **8b** showed high potentials as anti-inflammatory agents with no cytotoxicity.
- ✓ A single crystal X-ray structure of **5b** has been determined.

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