European Journal of Medicinal Chemistry 44 (2009) 5123–5130

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Short communication

2-Azetidinone derivatives: Design, synthesis, *in vitro* anti-microbial, cytotoxic activities and DNA cleavage study

Rangappa S. Keri, Kallappa M. Hosamani*, Ramya V. Shingalapur, Harisha R. Seetharama Reddy

P.G. Department of Studies in Chemistry, Karnatak University, Pavate Nagar, Dharwad 580 003, India

ARTICLE INFO

Article history:
Received 3 March 2009
Received in revised form
3 June 2009
Accepted 7 September 2009
Available online 11 September 2009

Keywords:
2-Azetidinone
Coumarin
Anti-microbial activity
Cytotoxicity
DNA cleavage

ABSTRACT

A novel series of 3-chloro-4-[4-(2-oxo-2*H*-chromen-4-ylmethoxy)-phenyl]-1-phenyl-azetidin-2-one derivatives (**5a–j**) have been synthesized from 4-aryloxymethylcoumarins (**1a–e**) and 4-aryliminomethylphenols (**3a–b**). The title compounds were screened for their *in vitro* anti-bacterial and anti-fungal activities. Results revealed that, compounds (**5c**), (**5f**), (**5h**) and (**5j**) showed excellent anti-microbial activity against a panel of microorganisms. Brine shrimp bioassay was also carried out to study their *in vitro* cytotoxic properties among which (**5h**) and (**5j**) displayed potent cytotoxic activity against *Artemia salina*. The DNA cleavage activity of some compounds was studied by agarose gel electrophoresis method. All synthesized compounds were characterized using IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analysis.

1. Introduction

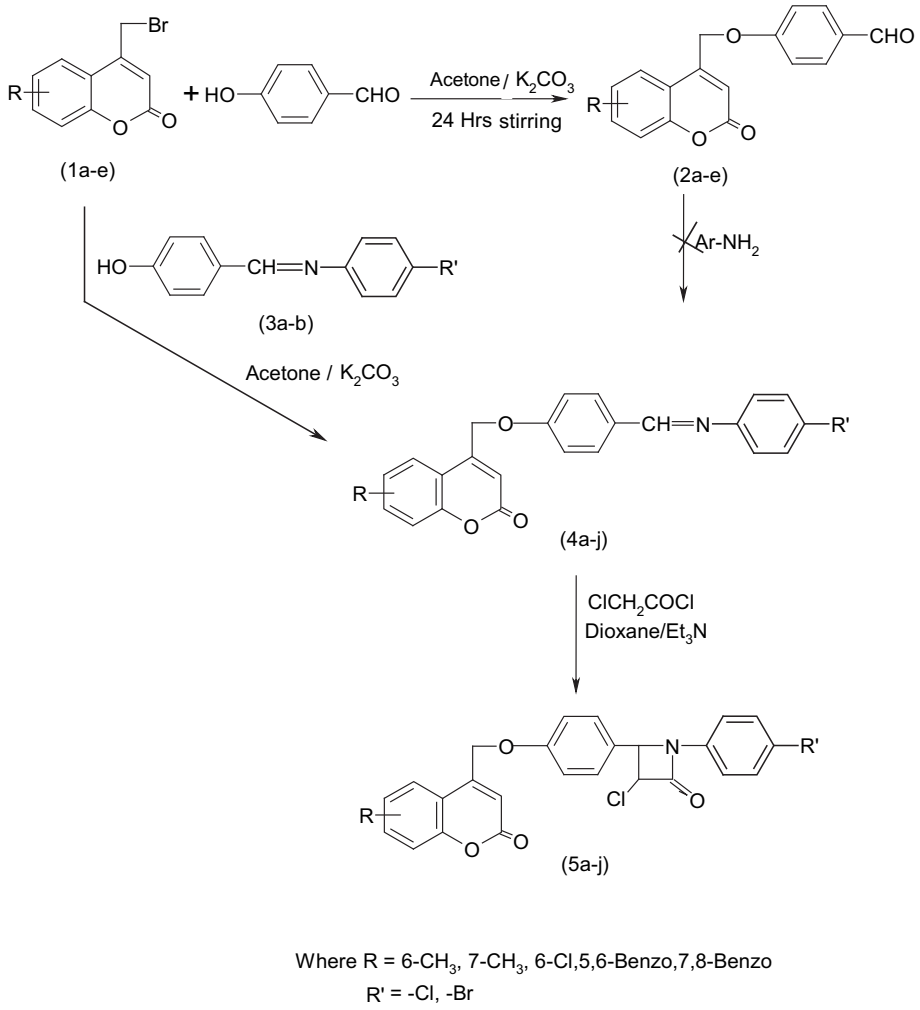
The discovery and development of new anti-microbial drugs in the search for better treatment has been a main goal for scientists. In recent decades, problems of multi-drug resistant microorganisms have reached an alarming level in many countries around the world. Resistance to a number of anti-microbial agents (β-lactam antibiotics, macrolides, quinolones and vancomycin) among a variety of clinically significant species of bacteria is becoming increasingly important global problem. Also a number of recent clinical reports describe the increasing occurrence of Methicillin resistant *Staphylococcus aureus* (MRSA) which is most disturbing cause of nosocomial infections in developed countries [1,2] and other antibiotic-resistant human pathogenic microorganisms in US and European Countries. Infections caused by these microorganisms pose a serious challenge to the medical community and the need for an effective therapy has led to the search for novel anti-microbial agents. In particular, increasing drug resistance among gram positive bacteria such as *staphylococci*, *enterococci* and *streptococci* is a significant health matter [3].

2-Azetidinones, commonly known as β-lactams are well-known heterocyclic compounds among organic and medicinal chemists [4]. The activity of famous antibiotics such as penicillins, cephalosporins, thienamycin, nocardicins, aztreonam and carbapenems are attributed to the presence of 2-azetidinone ring in them. Azetidinones are a very important class of compounds possessing a wide range of biological activities such as anti-microbial [5], anti-inflammatory [6], anti-convulsant [7], antibiotic [8], anti-cancer [9], anti-elastase [10], anti-viral [11], anti-tumor [5] and anti-HCMV [12] activities. Recently, it has been reported that β-lactams have novel biological activities such as cytomegalovirus protease inhibitors [13], thrombin and tryptase inhibitors [14], cholesterol absorption inhibitors [15], human leukocyte elastase (HLE) inhibitors [16], porcine pancreatic elastase (PPE) inhibitors [17], HIV-1 protease [18] and peroxisome proliferator-activated receptors (PPARs) [19]. Besides their biological activities, the importance of β-lactams as synthetic intermediates has been widely recognized in organic synthesis [20] for example in the semisynthesis of Taxol [21]. Like azetidinone, coumarin also exhibits diverse biological properties [22].

In the design of new drugs, the development of hybrid molecules through the combination of different pharmacophores in one frame may lead to compounds with interesting biological profiles. Owing to the importance and in continuation of our work on to the synthesis of biologically active compounds [23], now we wish to describe the synthesis of new azetidinone derivatives from 4-aryloxymethylcoumarins (Scheme 1) and *in vitro* screening results of anti-bacterial, anti-fungal, cytotoxic activities and DNA cleavage study. The other biological activities are under progress.

* Corresponding author. Tel.: +91 836 2215286; fax: +91 836 2747884/2771275.
E-mail address: dr_hosamani@yahoo.com (K.M. Hosamani).

0223-5234/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2009.09.011



Scheme 1. Schematic representation for the synthesis of azetidinone derivatives from 4-aryloxymethylcoumarin.

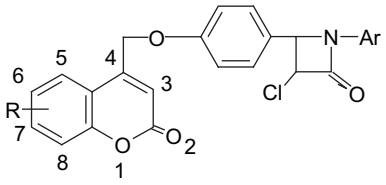
2. Chemistry

The present synthetic strategy begins with the generation of the required 4-bromomethylcoumarins (**1a**–**e**) by the Pechmann cyclisation of phenols with 4-bromoethylacetoacetate. Condensation of (**1a**) with 4-hydroxy benzaldehyde in presence of anhydrous K_2CO_3 yielded (2-oxo-2*H*-chromen-4-ylmethoxy) benzaldehydes (**2a**–**e**). In the IR Spectrum of 4-(2-oxo-2*H*-chromen-4-ylmethoxy)-benzaldehyde (**2a**), the lactone carbonyl stretching frequency was observed at 1718 cm^{-1}, whereas the aldehydic carbonyl stretching appeared at 1690 cm^{-1}. In the ^1H NMR spectrum of compound (**2a**), a singlet was observed at δ 2.38 ppm due to C_6–CH_3 protons. The C_4–CH_2 protons were observed downfield as a singlet at δ 5.31 ppm. The C_3–H of coumarin appeared at δ 6.64 ppm. The aldehydic proton appeared as a singlet at δ 9.95 ppm. The ^{13}C NMR spectral data of compound (**2a**) is given in the experimental section. In view of the poor reactivity of the *p*-carbonyl group observed in 4-aryloxymethylcoumarins, pre-functionalized phenols (**3a**–**b**) (obtained by the reaction of *p*-formyl phenol and aromatic amines) were used for room temperature allylic S_N reaction under standard acetone, K_2CO_3 conditions generating the required precursors (**4a**–**j**) in high yields. The IR spectrum of 6-methyl-4-(4-phenyliminomethyl-phenoxymethyl)-chromen-2-one (**4a**) (R = 6-CH_3, R_1 = H) showed lactone carbonyl stretching frequency at 1709 cm^{-1} and stretching frequency for C=N group at 1615 cm^{-1}. The PMR spectrum of (**4a**) exhibited a singlet due to –CH_3 protons at δ 2.24 ppm, C_4–CH_2 at δ 5.16 ppm and C_3–H at δ 6.33 ppm. The aromatic protons resonated in the range of δ 7.04–8.32 ppm. The azomethine proton appeared downfield as a singlet at δ 9.85 ppm. The azomethine group in (**4a**–**j**) underwent cycloaddition with chloroacetyl chloride in the presence of triethylamine [Et_3N] as a catalyst in dioxane afforded 3-chloro-4-[4-(2-oxo-2*H*-chromen-4-ylmethoxy)-phenyl]-1-phenyl-azetidin-2-ones (**5a**–**j**) (Scheme 1). The IR spectrum of 3-chloro-4-[4-(6-methyl-2-oxo-2*H*-chromen-4-ylmethoxy)-phenyl]-1-phenyl-azetidin-2-one (**5a**), the bands at 1764 cm^{-1} (>C=O of β-lactam) and coumarin carbonyl stretching at 1723 cm^{-1}. In the PMR spectra of compound (**5a**), the peak was observed at δ 5.29 ppm due to the >CH–Cl in the β-lactam ring; in ^{13}C NMR spectra of compound (**5a**), peaks at δ 57.3 ppm was observed due to >CH–Cl, 173.2 ppm (cyclic, >C=O) and 158.7 ppm (heteroaromatics) in the β-lactam moiety. The mass spectra of compound (**5a**) showed the molecular ion peak at 481 [M + 1]. The various new compounds synthesized during the present investigation are listed in Table 1.

3. Pharmacology

All the compounds (**5a**–**j**) prepared herein were screened for their *in vitro* anti-bacterial activity against two gram positive [*S. aureus* (ATCC-29213) and *Vancomycin resistant enteroccus* (ATCC-51299)] and two gram-negative [*Escherichia coli* (ATCC-25922) and *Shigella dysentery* (natural isolates)], *Aspergillus fumigatus* (ATCC-

Table 1
Physical and analytical data of the 2-azetidinone derivatives.



Entry	Product[a]	R	Ar	Yield[b] (%)	m.p.[c] (°C)	Mol. formula/mol. wt	Elem. analysis (Cal./found)		
							C	H	N
1	**5a**	6-CH_3	4-ClC_6H_4	73.35	174–176	$C_{26}H_{19}Cl_2NO_4$	65.01	3.99	2.92
						480	65.03	4.02	2.90
2	**5b**	7-CH_3	4-ClC_6H_4	69.93	132–133	$C_{26}H_{19}Cl_2NO_4$	65.01	3.99	2.92
						480	64.98	4.00	2.93
3	**5c**	6-Cl	4-ClC_6H_4	88.12	175–177	$C_{25}H_{16}Cl_3NO_4$	59.96	3.22	2.80
						500	60.00	3.25	2.78
4	**5d**	5,6-Benzo	4-ClC_6H_4	67.56	122–124	$C_{29}H_{19}Cl_2NO_4$	67.45	3.71	2.71
						516	67.41	3.74	2.69
5	**5e**	7,8-Benzo	4-ClC_6H_4	71.46	180–182	$C_{29}H_{19}Cl_2NO_4$	67.45	3.71	2.71
						516	67.43	3.68	2.73
6	**5f**	6-CH_3	4-BrC_6H_4	77.16	167–168	$C_{26}H_{19}BrClNO_4$	59.51	3.65	2.67
						524	59.55	3.63	2.65
7	**5g**	7-CH_3	4-BrC_6H_4	69.78	237–238	$C_{26}H_{19}BrClNO_4$	59.51	3.65	2.67
						524	59.49	3.68	2.70
8	**5h**	6-Cl	4-BrC_6H_4	64.68	159–160	$C_{25}H_{16}BrCl_2NO_4$	55.07	2.96	2.57
						545	55.05	2.97	2.60
9	**5i**	5,6-Benzo	4-BrC_6H_4	80.34	192–193	$C_{29}H_{19}BrClNO_4$	62.11	3.41	2.50
						560	62.07	3.44	2.53
10	**5j**	7,8-Benzo	4-BrC_6H_4	70.23	230–231	$C_{29}H_{19}BrClNO_4$	62.11	3.41	2.50
						560	62.14	3.40	2.47

[a] Products were characterized by IR, NMR, MS and elemental analysis.
[b] Isolated yields.
[c] Melting points are uncorrected.

36607), *Candida Albicans* (ATCC-10231) and *Penicillium* (natural isolates) as fungal strains were used for the *in vitro* study by the tube dilution technique [24]. The strains used in this study were maintained at the Department of Microbiology, Luqman Pharmacy College, Gulbarga. Each of the test compounds and standards, Ciprofloxacin and Gentamycin were dissolved in DMSO initially at 250 μg/ml and then were serially diluted in culture medium as follows: 125, 62.5, 31.250, 16, 8, 4, 2, and 1 μg/ml concentrations. The minimum inhibitory concentrations (MIC) were defined as the lowest concentrations of the compounds that prevented visible growth. It was determined that the solvent had no anti-bacterial activity against any of the test microorganisms. The brine shrimp bioassay was also carried out to study their *in vitro* cytotoxic activity against *A. salina*. The DNA cleavage of some 2-azetidinone derivatives was studied by agarose gel electrophoresis method.

4. Results and discussion

4.1. Anti-bacterial bioassay

MIC values for the *in vitro* anti-bacterial studies of the compounds (**5a**–**j**) and the standard are represented in Table 2. The

Table 2
Anti-microbial activity of the synthesized compounds (**5a**–**5j**).

MIC(μg/ml)							
Comp	Bacterial strains				Fungal strains		
	S. Aureus (29213)[a]	*V. r. enteroccoccus* (51299)	*E. coli* (25922)	*S. dysentery* (natural isolates)	*Aspergillus fumigatus* (36607)	*Candida albicans* (10231)[a]	*Penicillium* (natural isolates)
5a	08	16.0	04	08	16.0	16.0	16.0
5b	08	08	31.250	31.250	31.250	31.250	16.0
5c	01	02	01	01	08	08	16.0
5d	16.0	16.0	31.250	31.250	31.250	16.0	16.0
5e	31.250	62.5	62.5	62.5	16.0	16.0	16.0
5f	04	08	08	02	08	08	08
5g	08	04	08	08	16.0	16.0	08
5h	01	02	01	04	08	08	08
5i	16.0	31.250	62.5	16	31.250	31.250	31.250
5j	04	08	08	04	08	16	16
Ciprofloxacin	0.86	0.80	0.20	0.20	–	–	–
Gentamycin	–	–	–	–	10	10	10

[a] ATCC number.

Table 3
Brine shrimp bioassay data for the compounds (**5a**–**5j**).

Compound	LD_{50} (M)
5a	3.164×10^{-3}
5b	3.934×10^{-3}
5c	5.634×10^{-4}
5d	3.362×10^{-3}
5e	2.994×10^{-3}
5f	5.157×10^{-4}
5g	1.948×10^{-3}
5h	7.421×10^{-4}
5i	3.014×10^{-3}
5j	6.145×10^{-4}

anti-bacterial activity of all the compounds against *S. aureus*, *Vancomycin resistant enteroccus* as gram (+), *E. coli*, *S. dysentery* as gram (−) bacteria showed good potencies compared to control drug Ciprofloxacin. Among the synthesized compounds (**5c**), (**5f**), (**5h**) **and** (**5j**) showed very good activity with MIC value of 8–1 μg/ml. Compound (**5c**) showed excellent activity with an MIC of 1 μg/ml against all bacterial strains which is comparable with Ciprofloxacin. Compound (**5h**) exhibited MIC of 1 μg/ml against *S. Aureus* and 4 μg/ml against *S. dysentery*. Compound (**5f**) was also comparable with standard against *S. dysentery* with an MIC of 2 μg/ml. Particularly, compounds **5c** & **5h** with chloro substituent of coumarin moiety and bromo/chloro substituent at 4-position of phenyl ring of azetidinone moiety, have showed excellent activity with MIC value of 4–1 μg/ml. The other compounds (**5a**), (**5b**), (**5d**), (**5e**), (**5g**) and (**5i**) have exhibited moderate activity against all the bacterial strains.

4.2. Anti-fungal bioassay

The anti-fungal screening of all compounds were carried out against *A. fumigatus*, *C. albicans* and *Penicillium* fungal strains. The results were compared with that of standard drug Gentamycin. These results (Table 2) indicate that, the compounds (**5c**) and (**5f**), (**5h**) and (**5j**) exhibited promising activity against all the fungal strains. Compound (**5c**) was promising with an MIC of 8 μg/ml against both the fungi *A. fumigatus* and *C. albicans*. However, compounds (**5a**), (**5b**), (**5d**), (**5e**) and (**5g**) exhibited low to moderate activity against the fungal strains. In general, the high activity is attributed to the presence of electron withdrawing chloro and bromo functional groups as compared to other substituents.

4.3. Cytotoxic activity

All the synthesized compounds (**5a**–**j**) were screened for their cytotoxicity (brine shrimp bioassay) using the protocol of Meyer et al. [25]. From the data recorded in Table 3, it is evident that the compounds (**5c**), (**5f**), (**5h**) **and** (**5j**) displayed potent cytotoxic activity against *A. salina*, while the other compounds were almost inactive in this assay. Compound (**5h**) showed maximum activity ($LD_{50} = 7.421 \times 10^{-4}$ M) in the present series of compounds, whereas the other active compounds (**5c**), (**5f**) **and** (**5j**) demonstrated slightly less activity ($LD_{50} = 5.634 \times 10^{-4}$ M, 5.157×10^{-4} M and 6.145×10^{-4} M respectively) than compound (**5h**). The relationship between cytotoxicity and activity however, reveals that cytotoxicity is approximately 100-fold greater than concentration for the activity of the most active compound against the selected bacterial strains.

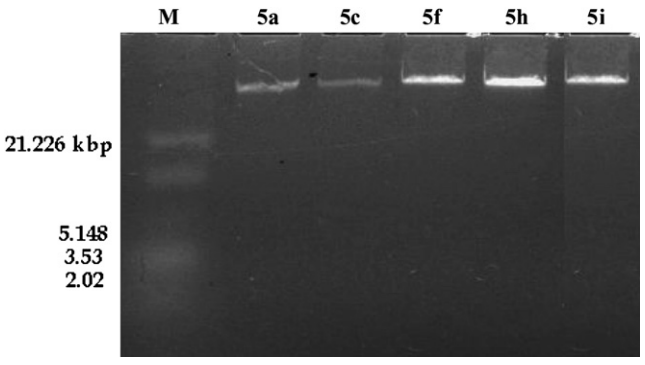


Fig. 1. DNA cleavage analysis of azetidinone derivatives- **5a**, **5c**, **5f**, **5h** and **5i**.

4.4. Electrophoretic analysis

The DNA cleavage of some 2-azetidinone derivatives was studied by agarose gel electrophoresis method and is presented in Fig. 1. The gel after electrophoresis clearly revealed that, the compound (**5i**) did acted on the DNA, as the streak was seen. The difference was observed in the bands of compounds (Lanes 1–5) compared to the control DNA of *E. coli*. This shows that the control DNA alone does not show any apparent cleavage as the compounds did. However, the nature of reactive intermediates involved in the DNA cleavage by the compounds has not been clear. The results indicate the importance of 2-azetidinone derivatives in these isolated DNA cleavage reactions. As the compound (**5i**) was observed to cleave the DNA, it can be concluded that the compound inhibits the growth of the pathogenic organism by cleaving the genome.

5. Conclusion

In conclusion, a series of 3-chloro-4-[4-(2-oxo-2*H*-chromen-4-ylmethoxy)-phenyl]-1-phenyl-azetidin-2-one derivatives were prepared (**5a**–**j**) and screened for their *in vitro* anti-bacterial activity against four strains of bacteria, anti-fungal activity against three strains of fungi and cytotoxic studies. Bioassay indicated that the compounds (**5c**), (**5f**), (**5h**) and (**5j**) were excellently active against all the strains and the remaining compounds showed good to moderate activity comparable with standard drugs. The compounds (**5c**), (**5h**) and (**5g**) displayed potent cytotoxic activity against *A. salina*. The DNA cleavage studies revealed that, 2-azetidinone derivative (**5i**) showed non-specific cleavage of DNA. On the basis of structure–activity relationship study of the compounds, it can be concluded that the presence of chlorine/bromine atom in the azetidinone moiety enhanced the activity of the compounds. Henceforth, our findings will have a good impact on chemists and biochemists for further investigations in this field in search of chlorine containing anti-microbial and cytotoxic agents.

6. Experimental protocol

6.1. Chemistry

Melting points of the synthesized compounds were determined in open capillaries and are uncorrected. Infrared spectra were recorded using KBr pellets on Nicolet 5700 FT-IR instrument. The ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker Avance-300 (300 MHz) model spectrophotometer in $CDCl_3$ and DMSO as solvent and TMSi as internal standard with ^{1}H resonant frequency of 300 MHz and ^{13}C resonant frequency of 75 MHz. The chemical shifts were measured in δ ppm downfield from internal standard TMSi at δ=0. The TLC was performed on alumina silica gel 60 F_{254} (Merck). The mobile phase was ethyl acetate and *n*-hexane (1:1)

and detection was made using UV light and iodine. The resulting compounds were purified by column chromatography. For column chromatography Merck silica gel (0.040–0.063 mm) was used. All the compounds gave C, H and N analysis within ±0.5% of the theoretical values.

6.2. General procedure for the preparation of compounds

6.2.1. Preparation of 4-bromomethylcoumarins (**1a–e**)

The present synthetic strategy begins with the generation of the required 4-bromomethylcoumarins (**1a–e**). These compounds were prepared by the Pechmann cyclization of phenols with 4-bromoethylacetoacetate [28,29].

6.2.2. Preparation of (2-oxo-2H-chromen-4-ylmethoxy)-benzaldehydes (**2a–e**)

p-Hydroxy benzaldehyde (1.24 g, 10 mmol) and anhydrous K_2CO_3 (1.38 g, 10 mmol) were stirred in dry acetone 25 ml for 30 min. 4-Bromomethylcoumarins (2.52 g, 10 mmol) were added and stirring was continued for 24 h. The reaction mixture was concentrated to one-fourth of the original volume and poured into ice-cold water. The solid separated was filtered and washed with 5% HCl (10 ml) to neutralize excess of potassium carbonate, then washed with 100 ml of cold water and with dilute ethanol. The crude product was dried and recrystallized from DMF.

*6.2.2.1. 4-(6-Methyl-2-oxo-2H-chromen-4-ylmethoxy)-benzaldehyde (**2a**).* Colourless solid, m.p.218–219 °C, IR (KBr υ cm^{-1}): 3046 (=CH–), 2778 (CH of CHO), 1718 (C=O of coumarin), 1690 (C=O of aldehyde), 1534 (C=C) cm^{-1}, 1022 (C–O–C); ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 2.38 (s, 3H, CH_3), 5.31 (s, 2H, CH_2O), 6.64 (s, 1H,C_3H), 7.12–7.97 (m, 7H, Ar–H), 9.95 (s, 1H, CHO); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 21.1, 72.1, 114.0, 118.0, 123.0, 133.1, 133.3, 148.2, 152.4, 161.0, 192.0; ESI-MS: 294 (M^+); Anal. Calcd for $C_{18}H_{14}O_4$: C, 73.45; H, 4.80; found C, 73.51; H, 4.87 %.

*6.2.2.2. 4-(7-Methyl-2-oxo-2H-chromen-4-ylmethoxy)-benzaldehyde (**2b**).* Colourless solid, m.p.223–225 °C, IR (KBr υ cm^{-1}): 3038 (=CH–), 2788 (CH of CHO), 1707 (C=O of coumarin), 1697 (C=O of aldehyde), 1562(C=C) cm^{-1}, 1016 (C–O–C); ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 2.48 (s, 3H, CH_3), 5.33 (s, 2H, CH_2O), 6.60 (s, 1H,C_3H), 7.15–7.91 (m, 7H, Ar–H), 9.91 (s, 1H, CHO); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 20.6, 78.0, 113.5, 117.8.0, 121.3, 134.1, 148.2, 155.4, 167.6, 188.0; ESI-MS: 294 (M^+); Anal. Calcd for $C_{18}H_{14}O_4$: C, 73.44; H, 4.81; found C, 73.48; H, 4.86%.

*6.2.2.3. 4-(6-Chloro-2-oxo-2H-chromen-4-ylmethoxy)-benzaldehyde (**2c**).* Colourless solid, m.p.220–222 °C, IR (KBr υ cm^{-1}): 3062 (=CH–), 2808 (CH of CHO), 1722 (C=O of Coumarin), 1698 (C=O of aldehyde), 1571 cm^{-1} (C=C), 1021 (C–O–C); ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 5.25 (s, 2H, CH_2O), 6.73 (s, 1H, C_3–H), 7.21–7.91 (m, 7H, Ar–H), 9.90 (s, 1H, CHO); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 83.4, 110.0, 116.0, 126.0, 135.1, 138.3, 148.2, 158.4, 161.0, 167.7, 192.0; ESI-MS: 315 (M + 1); Anal. Calcd for $C_{17}H_{11}ClO_4$: C, 64.87; H, 3.57, Cl, 11.28; found C, 64.91; H, 3.62, Cl, 11.32%.

*6.2.2.4. 4-(3-Oxo-3H-benzochromen-1-ylmethoxy)-benzaldehyde (**2d**).* Light yellow solid, m.p.222–224 °C, IR (KBr υ cm^{-1}): 3080 (=CH–), 2836 (CH of CHO), 1712 (C=O of coumarin), 1695(C=O of aldehyde), 1562 cm^{-1} (C=C), 1031 (C–O–C); ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 5.75 (s, 2H, CH_2O), 6.94 (s, 1H, C_3–H), 7.13–8.15 (m, 10H, Ar–H), 9.96 (s, 1H, CHO); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 81.4, 106.0, 114.0, 123.0, 126.8, 129.1, 130.3, 148.7, 163.5, 169.3, 193.2.; ESI-MS: 330 (M^+); Anal. Calcd for $C_{21}H_{14}O_4$: C, 76.38; H, 4.27; found C, 76.42; H, 4.32%.

*6.2.2.5. 4-(2-Oxo-2H-benzochromen-4-ylmethoxy)-benzaldehyde (**2e**).* Light reddish solid, m.p.248–249 °C, IR (KBr, υ cm^{-1}): 3052 (=CH–), 2842 (–CH of CHO), 1718 (C=O of coumarin), 1707 (C=O of aldehyde), 1569 cm^{-1} (C=C), 1028 (C–O–C); ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 5.48 (s, 2H, CH_2O), 6.80 (s, 1H, C_3–H), 7.08–8.89 (m, 10H, Ar–H), 9.92 (s, 1H, CHO); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 80.8, 107.5, 113.1, 122.1, 127.6, 129.8, 133.0, 146.6, 164.4, 168.8, 191.5.; ESI-MS: 330 (M^+); Anal. Calcd for $C_{21}H_{14}O_4$: C, 76.39; H, 4.26; found C, 76.45; H, 4.30%.

6.2.3. Preparation of 4-(4-phenyliminomethyl-phenoxymethyl)-chromen-2-ones **4(a–j)**

4-Phenyliminomethyl-phenols (**3a–b**) (10 mmol) and anhydrous K_2CO_3 (1.38 g, 10 mmol) were stirred in dry acetone (25 ml) for 30 min. 4-Bromomethylcoumarins (**1a–e**) (2.52 g, 10 mmol) was added and stirring was continued for 24 h. The reaction mixture was concentrated and poured into crushed ice (100 g). The solid separated was filtered and washed with 5% HCl (10 ml) to neutralize excess of potassium carbonate, then washed with 100 ml of cold water and with dilute ethanol. The crude product was dried and recrystallized from DMF.

*6.2.3.1. 4-{4-[(4-Chloro-phenylimino)-methyl]-phenoxymethyl}-6-methyl-chromen-2-one (**4a**).* Colourless crystals from DMF; Yield 69%; m.p. 244–245 °C; IR (KBr, υ cm^{-1}): 1709 (C=O of coumarin), 1615 (C=N); ^{1}H NMR (300 MHz, δ ppm, DMSO-d_6): 2.24 (s, 3H, C_6–CH_3), 5.16 (s, 2H, CH_2–O), 6.33 (s, 1H, C_3–H), 7.04–8.32 (m, 11H, Ar–H), 9.85 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 18.8, 81.4, 106.0, 113.6, 119.4, 122.8, 126.9, 128.9, 130.9, 134.6, 135.8, 146.8, 150.7, 156.6, 160.1, 162.3, 164.1; ESI-MS: 404 (M + 1); Anal. Calcd for $C_{24}H_{18}O_3NCl$: C, 71.38; H, 4.49, N, 3.47; found C, 71.40; H, 4.51, N, 3.50%.

*6.2.3.2. 4-{4-[(4-Chloro-phenylimino)-methyl]-phenoxymethyl}-7-methyl-chromen-2-one (**4b**).* Colourless crystals from DMF; Yield 69%; m.p. 257–258 °C; IR (KBr, υ cm^{-1}): 1715 (C=O of coumarin), 1606 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 2.31 (s, 3H, C_7–CH_3), 4.87 (s, 2H, CH_2–O), 6.32 (s, 1H, C_3–H), 6.88–7.87 (m, 11H, Ar–H), 9.89 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 20.1, 79.8, 107.9, 112.8, 118.8, 121.9, 125.3, 127.3, 129.7, 133.5, 137.8, 144.3, 149.8, 155.9, 159.8, 160.8, 166.5; ESI-MS: 404 (M + 1); Anal. Calcd for $C_{24}H_{18}O_3NCl$: C, 71.38; H, 4.49, N, 3.47; found C, 71.41; H, 4.52, N, 3.46%.

*6.2.3.3. 6-Chloro-4-{4-[(4-chloro-phenylimino)-methyl]-phenoxymethyl}-chromen-2-one (**4c**).* Colourless crystals from DMF; Yield 72 %; m.p. 214–216 °C; IR (KBr, υ cm^{-1}): 1721 (C=O of coumarin), 1612 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 4.59 (s, 2H, CH_2–O), 6.38 (s, 1H, C_3–H), 6.94–7.92 (m, 11H, Ar–H), 9.95 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 75.5, 109.1, 111.3, 116.9, 120.3, 124.7, 126.2, 129.3, 134.7, 136.3, 146.5, 147.5, 154.1, 157.4, 160.0, 162.3; ESI-MS: 425 (M + 1); Anal. Calcd for $C_{23}H_{15}O_3Cl_2N$: C, 65.11; H, 3.57; N, 3.30; found C, 65.13; H, 3.55, N, 3.33%.

*6.2.3.4. 4-{4-[(4-Chloro-phenylimino)-methyl]-phenoxymethyl}-benzo[f] chromen-3-one (**4d**).* Yellow crystals from DMF; Yield 72 %; m.p. 246–247 °C; IR (KBr, υ cm^{-1}): 1730 (C=O of coumarin), 1623 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 4.56 (s, 2H, CH_2–O), 6.29 (s, 1H, C_3–H), 6.78–7.85 (m, 14H, Ar–H), 9.08 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 80.2, 105.6, 113.4, 115.5, 116.5, 121.4, 123.5, 125.4, 127.5, 128.3, 129.9, 131.3, 149.3, 150.8, 155.7, 158.7, 161.2, 164.4; ESI-MS: 441 (M + 1); Anal. Calcd for $C_{27}H_{18}O_3ClN$: C, 73.72; H, 4.12; N, 3.18; found C, 73.74; H, 4.15, N, 3.15%.

*6.2.3.5. 4-{4-[(4-Chloro-phenylimino)-methyl]-phenoxymethyl}-benzo[h] chromen-2-one (**4e**).* Reddish crystals from DMF; Yield 65 %; m.p. 203–204 °C; IR (KBr, υ cm^{-1}): 1723 (C=O of coumarin), 1614 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 4.39 (s, 2H, CH_2–O), 6.43 (s, 1H, C_3–H), 6.86–7.81 (m, 14H, Ar–H), 9.32 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 78.5, 106.1, 111.9, 114.8, 118.2, 120.9, 122.9, 126.1, 127.7, 128.6, 130.4, 136.4, 147.5, 148.9, 155.3, 157.9, 160.3, 163.9; ESI-MS: 441 (M + 1); Anal. Calcd for $C_{27}H_{18}O_3ClN$: C, 73.72; H, 4.12; N, 3.18; found C, 73.74; H, 4.15, N, 3.15%.

*6.2.3.6. 4-{4-[(4-Bromo-phenylimino)-methyl]-phenoxymethyl}-6-methyl-chromen-2-one (**4f**).* Colourless crystals from DMF; yield 72 %; m.p. 241–242 °C; IR (KBr, υ cm^{-1}): 1717 (C=O of coumarin), 1603 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 1.93 (s, 3H, C_6–CH_3), 4.94 (s, 2H, CH_2–O), 6.09 (s, 1H, C_3–H), 6.87–7.95 (m, 11H, Ar–H), 9.53 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 20.2, 78.9, 108.4, 114.3, 118.3, 121.9, 125.3, 127.2, 129.8, 133.4, 137.6, 147.3, 151.3, 155.3, 158.5, 159.7, 162.0; ESI-MS: 449 (M + 1); Anal. Calcd for $C_{24}H_{18}O_3NBr$: C, 64.30; H, 4.05, N, 3.11; Found C, 64.27; H, 4.07, N, 3.13%.

*6.2.3.7. 4-{4-[(4-Bromo-phenylimino)-methyl]-phenoxymethyl}-7-methyl-chromen-2-one (**4g**).* Colourless crystals from DMF; yield 65%; m.p. 245–247 °C; IR (KBr, υ cm^{-1}): 1724 (C=O of coumarin), 1616 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 2.21 (s, 3H, C_6–CH_3), 5.18 (s, 2H, CH_2–O), 6.42 (s, 1H, C_3–H), 7.00–8.25 (m, 11H, Ar–H), 9.17 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 19.7, 80.5, 106.5, 113.8, 117.6, 122.7, 126.7, 128.3, 130.7, 135.8, 138.9, 148.9, 153.2, 156.2, 158.8, 159.2, 161.4; ESI-MS: 449 (M + 1); Anal. Calcd for $C_{24}H_{18}O_3NBr$: C, 64.30; H, 4.05, N, 3.11; Found C, 64.29; H, 4.09, N, 3.11%.

*6.2.3.8. 4-{4-[(4-Bromo-phenylimino)-methyl]-phenoxymethyl]-6-chloro-chromen-2-one (**4h**).* Colourless crystals from DMF; yield 68%; m.p. 231–232 °C; IR (KBr, υ cm^{-1}): 1718 (C=O of coumarin), 1608 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): = 4.67 (s, 2H, CH_2–O), 6.29 (s, 1H, C_3–H), 6.77–7.89 (m, 11H, Ar–H), 9.78 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 77.9, 104.8, 112.6, 114.8, 119.7, 123.8, 125.8, 130.5, 133.5, 138.6, 144.7, 148.9, 153.8, 156.9, 159.7, 160.9; ESI-MS: 425 (M + 1); Anal. Calcd for $C_{23}H_{15}O_3BrClN$: C, 58.95; H, 3.25; N, 3.00; Found C, 58.97; H, 3.22, N, 3.03%.

*6.2.3.9. 1-{4-[(4-Bromo-phenylimino)-methyl]-phenoxymethyl}-benzo[f] chromen-3-one (**4i**).* Yellow crystals from DMF; Yield 81%; m.p. 177–179 °C IR (KBr, υ cm^{-1}): 1726(C=O of coumarin), 1609 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 4.66 (s, 2H, CH_2–O), 6.42 (s, 1H, C_3–H), 6.89–7.99 (m, 14H, Ar–H), 9.76 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 76.8, 107.2, 114.6, 116.9, 118.9, 120.3, 122.7, 124.8, 126.9, 127.7, 130.0, 133.7, 147.5, 151.7, 153.9, 157.9, 158.0, 163.3; ESI-MS: 485 (M + 1); Anal. Calcd for $C_{27}H_{18}O_3BrN$: C, 66.95; H, 3.75; N, 2.89; Found C, 66.98; H, 3.78, N, 2.92%.

*6.2.3.10. 4-{4-[(4-Bromo-phenylimino)-methyl]-phenoxymethyl}-benzo[h] chromen-2-one (**4j**).* Reddish crystals from DMF; yield 72%; m.p. 237–238 °C; IR (KBr, υ cm^{-1}): 1731 (C=O of coumarin), 1601 (C=N); ^{1}H NMR (300 MHz, δ ppm DMSO-d_6): 4.61 (s, 2H, CH_2–O), 6.32 (s, 1H, C_3–H), 6.94–7.92 (m, 14H, Ar–H), 9.64 (s, 1H, –CH=N) ppm; ^{13}C NMR (75 MHz, δ ppm, DMSO-d_6): 80.3, 107.7, 110.1, 113.3, 116.0, 119.3, 123.3, 125.4, 126.8, 129.2, 131.9, 138.2, 145.3, 151.3, 153.4, 154.7, 157.7, 162.5; ESI-MS: 485 (M + 1); Anal. Calcd for $C_{27}H_{18}O_3BrN$: C, 66.95; H, 3.75; N, 2.89; Found C, 66.93; H, 3.77, N, 2.93%.

*6.2.4. Preparation of 3-chloro-4-[4-(2-oxo-2H-chromen-4-ylmethoxy)-phenyl]-1-phenyl-azetidin-2-ones (**5a**–**j**)*

The mixture of 4-(4-Phenyliminomethyl-phenoxymethyl)-chromen-2-ones (0.01 mol) and triethylamine (0.01 mol) was dissolved in dioxane (50 ml), cooled and stirred. To this well-stirred cold solution of chloroacetyl chloride (0.01 mmol) was added drop wise within a period of 20 min. The reaction mixture was then stirred for an additional 3 h and left at room temperature for 48 h. The resultant mixture was concentrated, cooled, poured into ice cold water, filtered and then dried. The product thus obtained was purified by column chromatography over silica gel using 30% ethyl acetate: 70% benzene as an eluent. Recrystallization was done from suitable solvent which gave 2-azetidinones (**5a**–**j**).

*6.2.4.1. 3-Chloro-1-[(4-chloro-phenyl)-4-[4-(6-methyl-2-oxo-2H-chromen-4-ylmethoxy)-phenyl]-azetidin-2-one] (**5a**).* Colourless shiny crystals from DMF, m.p. 174–176 °C, IR (KBr, υ cm^{-1}): 3107 (=CH–), 1764 (>C=O of β-lactam), 1723 (>C=O of coumarin), 1503 (C=C), 782 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 2.22 (s, 3H, C_6–CH_3), 4.63 (s, 2H, CH_2–O), 4.95 (s, 1H, –N–CH), 5.29 (s, 1H, –CH–Cl), 6.21 (s, 1H, C_3–H), 6.67–7.92 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 17.9, 57.3, 61.0, 63.7, 80.2, 104.2, 111.2, 119.3, 120.4, 126.3, 129.3, 130.5, 140.4, 147.1, 157.0, 158.7, 162.1, 173.2; ESI-MS: 481 (M + 1).

*6.2.4.2. 3-Chloro-1-[(4-chloro-phenyl)-4-[4-(7-methyl-2-oxo-2H-chromen-4-ylmethoxy)-phenyl]-azetidin-2-one] (**5b**).* Colourless shiny crystals from DMF, m.p. 132–133 °C, IR (KBr, υ cm^{-1}): υ 3089 (=CH–), 1758 (>C=O of β-lactam), 1703 (>C=O of coumarin), 1489 (C=C), 780 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 2.03 (s, 3H, C_7–CH_3), 4.44 (s, 2H, CH_2–O), 5.12 (s, 1H, –N–CH), 5.42 (s, 1H, –CH–Cl), 5.89 (s, 1H, C_3–H), 6.76–7.79 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 21.0, 57.3, 63.7, 81.2, 102.3, 113.0, 116.8, 121.0, 125.7, 127.8, 132.6, 139.3, 145.9, 156.2, 157.5, 160.4, 167.5; ESI-MS: 481 (M + 1).

*6.2.4.3. 3-Chloro-4-[4-(6-chloro-2-oxo-2H–chromen-4-ylmethoxy)-phenyl]-1-(4-chloro-phenyl)–azetidin-2-one (**5c**).* Colourless shiny crystals from DMF, m.p. 175–177 °C, IR (KBr, υ cm^{-1}): 3089 (=CH–), 1771 (>C=O of β-lactam), 1718 (>C=O of coumarin), 1497 (C=C), 791 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.12 (s, 2H, CH_2–O), 4.79 (s, 1H, –N–CH), 5.19 (s, 1H, –CH–Cl), 6.09 (s, 1H, C_3–H), 6.59–7.76 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 53.8, 61.2, 79.3, 107.3, 112.0, 120.3, 122.6, 125.7, 127.2, 129.9, 133.3, 137.3, 146.8, 155.7, 159.8, 163.6, 179.6; ESI-MS: 502 (M + 2).

*6.2.4.4. 3-Chloro-1-(4-chloro-phenyl)-4-[4-(3-oxo-3H-benzo[f]chromen-1-ylmethoxy)-phenyl]- azetidin-2-one (**5d**).* Light brown crystals from DMF, m.p. 122–124 °C, IR (KBr, υ cm^{-1}): υ 3133 (=CH–), 1773 (>C=O of β-lactam), 1725 (>C=O of coumarin), 1506 (C=C), 785 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.34 (s, 2H, CH_2–O), 4.78 (s, 1H, –N–CH), 5.30 (s, 1H, –CH–Cl), 6.19 (s, 1H, C_3–H), 6.64–7.83 (m, 14H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 57.6, 64.1, 80.4, 104.2, 111.8, 115.2, 116.4, 120.1, 124.3, 126.4, 127.7, 130.5, 133.4, 134.6, 139.4, 150.9 156.7, 160.1, 165.7, 171.0; ESI-MS: 518 (M + 2).

*6.2.4.5. 3-Chloro-1-(4-chloro-phenyl)-4-[4-(2-oxo-2H-benzo[h]-chromen-4-ylmethoxy)-phenyl]- azetidin-2-one (**5e**).* Light reddish crystals from DMF, m.p. 180-182 °C, IR (KBr, υ cm^{-1}): 3088 (=CH–), 1767 (>C=O of β-lactam), 1718 (>C=O of coumarin), 1478 (C=C), 779 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.56 (s, 2H, CH_2–O), 4.91 (s, 1H, –N–CH), 5.41 (s, 1H, –CH–Cl), 6.37 (s, 1H, C_3–H), 6.71–7.92 (m, 14H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 53.9, 62.6, 78.3, 102.4, 110.1, 113.9, 115.3, 119.7, 123.5, 125.0, 127.0, 130.8,

132.7, 135.7, 140.7, 147.7 154.5, 157.7, 160.1, 168.3; ESI-MS: 518 (M + 2).

*6.2.4.6. 1-(4-Bromo-phenyl)-3-chloro-4-[4-(6-methyl-2-oxo-2H–chromen-4-ylmethoxy)-phenyl]-azetidin-2-one (**5f**).* Colourless shiny crystals from DMF, m.p. 167–168 °C, IR (KBr, υ cm^{-1}): 3089 (=CH–), 1756 (>C=O of β-lactam), 1713 (>C=O of coumarin), 1457 (C=C), 775 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 1.78 (s, 3H, C_6–CH_3), 4.57 (s, 2H, CH_2–O), 4.88 (s, 1H, –N–CH), 5.38 (s, 1H, –CH–Cl), 6.09 (s, 1H, C_3–H), 6.85–7.68 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 20.4, 60.2, 64.6, 77.5, 106.7, 113.9, 116.7, 123.7, 127.7, 129.9, 133.6, 138.9, 147.7, 158.4, 160.1, 161.7, 167.6; ESI-MS: 525 (M + 1).

*6.2.4.7. 1-(4-Bromo-phenyl)-3-chloro-4-[4-(7-methyl-2-oxo-2H–chromen-4-ylmethoxy)-phenyl]-azetidin-2-one (**5g**).* Colourless shiny crystals from DMF, m.p. 237–238 °C, IR (KBr, υ cm^{-1}): 3105 (=CH–), 1771 (>C=O of β-lactam), 1722 (>C=O of coumarin), 1418 (C=C), 779 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 1.92 (s, 3H, C_7–CH_3), 4.39 (s, 2H, CH_2–O), 5.06 (s, 1H, –N–CH), 5.27 (s, 1H, –CH–Cl), 6.22 (s, 1H, C_3–H), 6.76–7.79 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 21.5, 59.7, 63.3, 82.0, 106.7, 110.7, 117.8, 121.6, 126.7, 128.8, 131.7, 141.6, 146.8, 155.8, 159.1, 160.0, 164.5; ESI-MS: 525 (M + 1).

*6.2.4.8. 1-(4-Bromo-phenyl)-3-chloro-4-[4-(6-chloro-2-oxo-2H–chromen-4-ylmethoxy)-phenyl]-azetidin-2-one (**5h**).* Colourless shiny crystals from DMF, m.p.159–160 °C, IR (KBr, υ cm^{-1}): 3078 (=CH–), 1749 (>C=O of β-lactam), 1712 (>C=O of coumarin), 1444 (C=C), 789 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.34 (s, 2H, CH_2–O), 4.93 (s, 1H, –N–CH), 5.32 (s, 1H, –CH–Cl), 6.26 (s, 1H, C_3–H), 6.76–7.78 (m, 11H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 57.9, 60.9, 83.1, 106.8, 114.6, 119.6, 121.7, 124.6, 126.9, 130.5, 132.6, 138.2, 143.7, 153.9, 158.1, 160.4, 164.9; ESI-MS: 547 (M + 2).

*6.2.4.9. 1-(4-Bromo-phenyl)-3-chloro–4-[4-(3-oxo-3H-benzo[f]chromen-1-ylmethoxy)-phenyl]-azetidin-2-one (**5i**).* Light yellow crystals from DMF, m.p.192–193 °C, IR (KBr, υ cm^{-1}): 3110 (=CH–), 1766 (>C=O of β-lactam), 1720 (>C=O of coumarin), 1454 (C=C), 779 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.60 (s, 2H, CH_2–O), 5.13 (s, 1H, –N–CH), 5.37 (s, 1H, –CH–Cl), 6.35 (s, 1H, C_3–H), 6.61–7.76 (m, 14H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 58.5, 61.9, 78.9, 106.5, 113.9, 114.6, 115.9, 119.6, 124.2, 125.9, 127.9, 131.6, 133.0, 134.9, 140.8, 150.3, 155.2, 159.5, 160.5, 164.3; ESI-MS: 562 (M + 2).

*6.2.4.10. 1-(4-Bromo-phenyl)-3-chloro–4-[4-(3-oxo-3H-benzo[h]-chromen-1-ylmethoxy)-phenyl]-azetidin-2-one (**5j**).* Light brown crystals from DMF, m.p.230–231 °C, IR (KBr, υ cm^{-1}): 3093 (=CH–), 1757 (>C=O of β-lactam), 1722 (>C=O of coumarin), 1467 (C=C), 787 (–C–Cl) cm^{-1}; ^{1}H NMR (300 MHz, δ ppm, $CDCl_3$): 4.25 (s, 2H, CH_2–O), 5.14 (s, 1H, –N–CH), 5.31 (s, 1H, –CH–Cl), 6.32 (s, 1H, C_3–H), 6.73–7.88 (m, 14H, Ar–H); ^{13}C NMR (75 MHz, δ ppm, $CDCl_3$): 55.9, 60.7, 79.9, 103.9, 112.0, 113.6, 115.8, 118.9, 122.1, 125.7, 126.9, 129.7, 132.5, 135.6, 138.6, 146.7 155.9, 159.6, 160.4, 163.2; ESI-MS: 562 (M + 2).

6.3. Pharmacology

6.3.1. Anti-bacterial assay

The cultures were obtained in Mueller–Hinton Broth (Difco) for all the bacteria after 18–24 h of incubation at 37 ± 1 °C. Testing was carried out in Mueller–Hinton Broth at pH 7.4 and two-fold dilution technique was applied. A set of tubes containing only inoculated broth was kept as controls. After incubation for 18–24 h at 37 ± 1 °C, the last tube with no growth of microorganism was recorded to represent MIC expressed in μg/ml. Ciprofloxacin was used as standard drug.

6.3.2. Anti-fungal assay

The yeasts were maintained in Sabouraud Dextrose Broth (Difco) after incubation for 48 h at 25 ± 1 °C. Testing was performed in Sabouraud Dextrose Broth at pH 7.4 and the twofold dilution technique was applied. A set of tubes containing only inoculated broth was kept as controls. After incubation for 48 h at 25 ± 1 °C, the last tube with no growth of yeast was recorded to represent MIC expressed in μg/ml. Gentamycin was used as standard drug.

6.3.3. Cytotoxic activity

Brine shrimp (*A. salina* leach) eggs were hatched in a shallow rectangular plastic dish (22 × 32 cm), filled with artificial sea water, which was prepared with commercial salt mixture and double distilled water. An unequal partition was made in the plastic dish with the help of a perforated device. Approximately 50 mg of eggs were sprinkled into the large compartment, which was darkened while the other compartment was opened to ordinary light. After two days nauplii were collected by a pipette from the lighted side. A sample of the test compound was prepared by dissolving 20 mg of each compound in 2 ml of DMF. From this stock solutions 500, 50 and 5 μg/ml were transferred to 9 vials (three for each dilutions were used for each test sample and LD_{50} is the mean of three values) and one vial was kept as control having 2 ml of DMF only. The solvent was allowed to evaporate overnight. After two days, when shrimp larvae were ready, 1 ml of sea water and 10 shrimps were added to each vial (30 shrimps/dilution) and the volume was adjusted with sea water to 5 ml per vial. After 24 h the number of survivors was counted [25]. Data were analyzed by a Finney computer program to determine the LD_{50} values [26].

6.3.4. DNA cleavage experiment

6.3.4.1. Preparation of culture media. DNA cleavage experiments were done according to the literature [27]. Nutrient broth [peptone, 10; Yeast extract, 5; NaCl, 10; in (g/l)] was used for culturing of *E. coli*. 50 ml media was prepared, autoclaved for 15 min at 121 °C under 15 lb pressure. The autoclaved media were inoculated for 24 h at 37 °C.

6.3.4.2. Isolation of DNA. The fresh bacterial culture (1.5 ml) is centrifuged to obtain the pellet which is then dissolved in 0.5 ml of lysis buffer (100 mM tris pH 8.0, 50 mM EDTA, 10% SDS). To this 0.5 ml of saturated phenol was added and incubated at 55 °C for 10 min, then centrifuged at 10,000 rpm for 10 min and to the supernatant, equal volume of chloroform: isoamyl alcohol (24:1) and 1/20th volume of 3 M sodium acetate (pH 4.8) was added. Again centrifuging at 10,000 rpm for 10 min and to the supernatant, 3 volumes of chilled absolute alcohol were added. The precipitated DNA was separated by centrifugation and the pellet was dried and dissolved in TAE buffer (10 mM tris pH 8.0, 1 mM EDTA) and stored in cold condition.

6.3.4.3. Agarose gel electrophoresis. Cleavage products were analyzed by agarose gel electrophoresis method [27]. Test samples (1 mg/ml) were prepared in DMF. The samples (25 mg) were added to the isolated DNA of *E. coli*. The samples were incubated for 2 h at 37 °C and then 20 ml of DNA sample (mixed with bromophenol blue dye at 1:1 ratio) was loaded carefully into the electrophoresis chamber wells along with standard DNA marker containing TAE buffer (4.84 g tris base, pH 8.0, 0.5 M EDTA/1 L) and finally loaded on agarose gel and passed the constant 50 V of electricity for around 30 min. Removing the gel and being stained with 10 .0 mg/ml ethidium bromide for 10–15 min, the bands were observed under Vilberlourmate Gel documentation system and then

photographed to determine the extent of DNA cleavage. Henceforth the results were compared with standard DNA marker.

Acknowledgments

This research work is financially supported by the Council of Scientific & Industrial Research (CSIR), New Delhi 110 012. (Ref: No. 01(2301)/09/EMR-II dated 19-03-2009).

References

[1] J.S. Francis, M.C. Doherty, U. Lopatin, C.P. Johnston, G. Sinha, T. Ross, M. Cai, N.N. Hanse, T. Per, J.R. Ticehurst, K. Carroll, D.L. Thomas, E. Nuermberger, J.G. Barlett, Clin. Infect. Dis. 40 (2005) 100–107.
[2] D. Kruszewska, H.G. Sahl, G. Bierbaum, U. Pag, S.O. Hynes, A. Ljungh, J. Antimicrob, Chemotherapy 54 (2004) 648–653.
[3] T.W. Chu, J.J. Plattner, L. Katz, J. Med. Chem. 36 (1996) 3853–3874.
[4] G.I. Georg, in: The Organic Chemistry of β-Lactams, VCH, New York, 1993.
[5] G.S. Singh, B.J. Mmolotsi, IL Farmaco 60 (9) (2005) 727–730.
[6] Ashok Kumar, C.S. Rajput, S.K. Bhati, Bioorg. Med. Chem. 15 (2007) 3089–3096.
[7] J.S. Biradar, S.Y. Manjunath, Indian J. Chem. B 43B (2004) 141–143.
[8] J.M. Desai, V.H. Shah, Indian J. Chem. B 42B (2003) 631–635.
[9] B.K. Banik, F.F. Becker, I. Banik, Bioorg. Med. Chem. 12 (10) (2004) 2523–2528.
[10] J. Anaya, D.S. Gero, M. Grande, J.I.M. Hernando, N.M. Laso, Bioorg. Med. Chem. 7 (5) (1999) 837–850.
[11] G. Gerona-Navarro, M.J. Perez de Vega, M.T. Garcia-Lopez, G. Andrei, R. Snoeck, E. De Clercq, J. Balzarini, R. Gonzalez-Muniz, J. Med. Chem. 48 (7) (2005) 2612–2621.
[12] C. Yoakim, W.W. Ogilvie, D.R. Cameron, C. Chabot, I. Guse, B. Haché, J. Naud, J.A. O'Meara, R. Plante, R. Déziel, J. Med. Chem. 41 (1998) 2882–2891.
[13] J.C. Powers, J.L. Asgian, O.D. Ekici, K.B.E. James, Chem. Rev. 102 (2002) 4639–4750.
[14] M. Bengalia, R. Annunziata, M. Clinquini, F. Cozzi, J. Org. Chem. 68 (2003) 2952–2955.
[15] C.M.L. Delpiccolo, E.G. Mata, Tetrahedron Lett. 45 (2004) 4085–4088.
[16] S. Gerard, G. Dive, B. Clamet, R. Touillaux, J. Marchand-Brynaert, Tetrahedron 58 (2002) 2423–2433.
[17] W. Bode, E.F. Meyer, J.C. Powers, Biochemistry 28 (1989) 1951–1963.
[18] J. Tozsera, T. Sperka, J. Pitlik, P. Bagossia, Bioorg. Med. Chem. Lett. 15 (2005) 3086–3090.
[19] Wei Wang, Pratik Devasthale, Dennis Farrelly, Gu Liqun, Bioorg. Med. Chem. Lett. 18 (2008) 1939–1944.
[20] B. Alcaide, P. Almendros, Synlett (2002) 381–393.
[21] D. Castagnolo, S. Armaroli, F. Corelli, M. Botta, Tetrahedron: Asymmetry 15 (6) (2004) 941–949.
[22] D. Eagn, R.O. Kennedy, Drug Metab. Rev. 22 (1990) 503–529.
[23] K.M. Hosamani, R.S. Harisha, R.S. Keri, S.H. Manohar, M.G. Moloney, J. Enz. Inhib. Med. Chem., doi:10.1080/14756360802632716. in press.
[24] D.F. Sahm, J.A. Washington, in: A. Balowes, W.J. Hausler, K.L. Hermann, H.D. Shadomy (Eds.), Antibacterial Suspectibility Tests, Dilution Methods, fifth ed. American Society for Microbiology, Washington, 1991, pp. 1105–1116.
[25] B.N. Meyer, N.R. Ferrigni, J.E. Putnam, L.B. Jacobsen, D.E. Nichols, J.L. McLaughlin, Planta Med. 45 (1982) 31–34.
[26] D.J. Finney, Probit. Analysis, Third ed. Cambridge University Press, Cambridge, 1971.
[27] T.A. Brown, in: Essential Molecular Biology - A Practical Approach, Oxford University Press, Great Clarendon Street, Oxford-OX 26DP, UK, Vol 1, 1990, pp. 51–52.
[28] M.V. Kulkarni, B.G. Pujar, V.D. Patil (Weinheim). Arch. Pharmacol. 316 (2) (1983) 15–21.
[29] A. Burger, G.E. Ullyot, J. Org. Chem. 12 (1947) 346–355.