Synthesis, characterization, industrial application and anticancer activity of new N-mustard substituted coumarin derivatives

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Abstract
A series of N-mustard Substituted-coumarin have been synthesized characterized and evaluated for their in vitro cytotoxicity and anticancer activity against PC-3 human cancer cell lines. The methodology involves nucleophilic substitution of readily accessible 3-Cyano-4-chloro coumarin with ethanol amine further on chlorination gives substituted 4-(bis(2-chloroethyl)amino)-2-oxo-2H-chromene-3-carbonitrile derivatives. Among these compounds screened, three compounds (ASW-2g, ASW-2f and ASW-2e) showed GI_{50} range from 80 to 95 µg/ml. All synthesized compounds were characterized by IR, NMR and Mass spectral analysis and screened for anticancer activity using PC-3 cell line. All the compounds showed moderate to good anticancer activity.

Keywords: *4-chloro-3-cyano coumarin, Aniline mustard, Anticancer activity, Nucleophilic substitution, Spectral analysis*

Introduction

The N-mustards were among the very earliest class of anticancer agents developed, and perhaps most extensively studied of the DNA alkylating agents[1]. Alkylation of DNA can then take place via nucleophilic attack on that intermediate by DNA[2]. For N-mustards, the regiospecificity of alkylation of DNA is largely governed by electronic and stearic properties of DNA. Therefore, they target DNA at the most electronegative sites, with mono adducts occurring primarily at the N-7 of guanines[3] and the interstrand cross-links between the N-7 positions of guanines in each strand at 5'-GNC sequences[4]. The present work will serve number of novel N-nitrogen mustard containing functionalized coumarin as potent antitumor agents using novel synthetic approaches. Coumarin and its hydroxyl derivatives have been prominently accepted as natural pharmaceuticals[5] worldwide, has revealed new biological activities with interesting therapeutic applications, besides their traditional employment as anticoagulants(anti-vitamin K activity)[6], antibiotic[7] and anti AID[8]. Apart from this, they also possess anti-cancerous[9], antibacterial[10], neurotropic[11], anti-inflammatory[12].

Experimental

All chemicals and solvents were purchased from Spectrochem Pvt Ltd., Mumbai of AR grade and were used without further purification. Melting points were taken in open capillary method and are uncorrected. IR spectra were recorded on FTIR-8400 spectrophotometer (Shimadzu, Kyoto, Japan), using DRS probe KBr pallet. ^{1}H-NMR spectra of the synthesized compounds were recorded on a Bruker-Avance-II (400 MHz) DMSO-*d*6 solvent.
Chemical shifts are expressed in δ ppm downfield from TMS as an internal standard. Mass spectra were determined using direct inlet probe on a GCMS-QP 2010 mass spectrometer (Shimadzu, Kyoto, Japan). Physical constants of the synthesized compounds ASW2a to ASW2l are shown in Table 1.

Synthesis of 4-hydroxy coumarin (int-1)

Various Substituted phenols (0.1 mole) and malonic acid were added to a mixture of phosphorus oxychloride (40 ml) and anhydrous zinc chloride (30 gm) which was preheated to get rid of any moisture. The reaction mixture was heated on a water bath at 700°C for 8-10 hours. It was cooled and decomposed with ice and water to afford buff-yellow coloured solid. The solid was then filtered and washed thoroughly with water. It was then triturated with 10% sodium carbonate solution and filterd. The filtrate was slowly acidified with dilute HCl till the effervescence ceased. The product was filtered, dried and recrystallized with methanol.

Synthesis of 4-chloro-3-formayl coumarin (int-2)

To a stirred mixture of 4-hydroxycoumarin **(**0.06 mole) in anhydrous DMF (0.6 mole) were added dropwise $POCl_3$ (0.18 mole) at –10°C to –5°C. The reaction mixture was then stirred for 1 hr at room temperature and heated and stirred for 2 hr at 60°C.After the reaction completed, the mixture was poured onto crushed ice under vigorous stirring. After stirring the mixture overnight at 0°C the pale yellow solid was collected by filtration and washed successively with Na_2CO_3 (5%) and water, and then was air–dried. Recrystallization from acetone gave 85% of 4-chloro-3-formyl coumarin as apale yellow powder with m.p. 115–120 °C.

Synthesis of 4-chloro-3-cyano coumarin (int-3)

To a 20 mL solution of 4-chloro-3-formylcoumarin (0.01 mole) in glacial acetic acid was added sodium acetate (0.01mole) and hydroxyl amine hydrochloride (0.01 mole) and the solution was allowed to stir at 60°C for 2 hr. After completion of the reaction monitored the solid was filtered and washed with water (25 ml). Crystallization of compound from DMF:IPA (80:20) under 0-5°C yields 45% of 4-chloro-3-cyano coumarin as a light green crystals m.p. 198–200 °C.

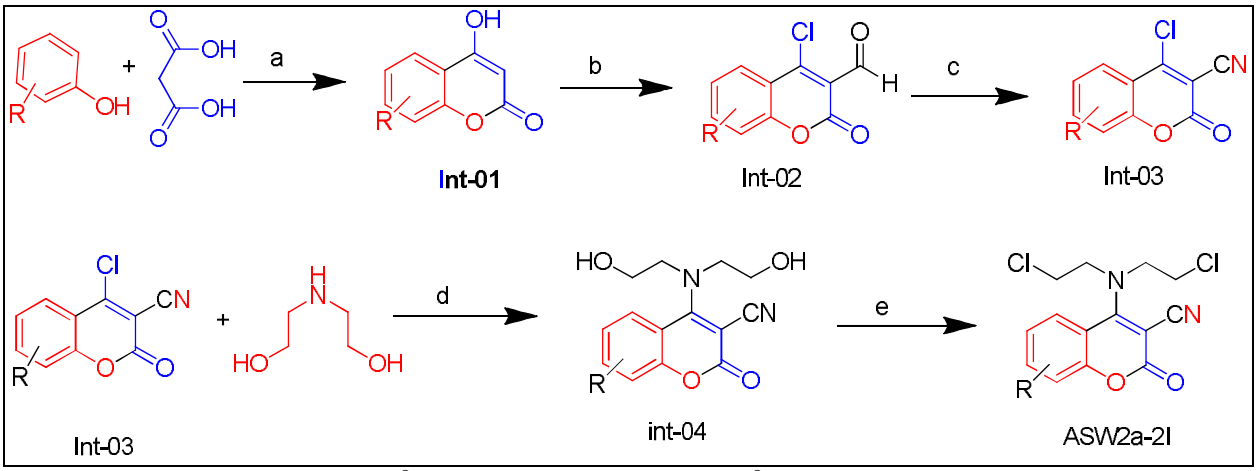
4-(bis(2-hydroxyethyl)amino)-2-oxo-2H-chromene-3-carbonitrile (int-4)

To a solution of DMF and 4-chloro-3-carbonitril coumarin (0.01 mole),thionyl chloride(0.01mole) added drop wise at 0°C. After the completion of addition, reaction mixture was allowed to stir room temperature for further 2 hr. After completion of the reaction, mixture was poured onto crushed ice under vigorous stirring the solid was filtered and washed with water.

General synthesis of substituted4-(bis(2-chloroethyl)amino)-2-oxo-2H-chromene-3-carbonitrile derivatives (ASW-2a-2l)

4-chloro-3-cyano coumarin (0.01 mole) was dissolved in 10 mL IPA and allowed to stir between 0-5°C followed by addition of diethanolamine (0.2mole) was carefully added to the solution so that the temperature do not rise 10°C. Allow it to stir for 30 min. and slowly rise to room temperature. After completion of the reaction, was poured into crushed ice, filtered and washed with water. Crystallization from chloroform gives 4-substituted-2-oxo-2*H*-chromene-3-carbonitirles. Yield 61-88%.

Reaction scheme



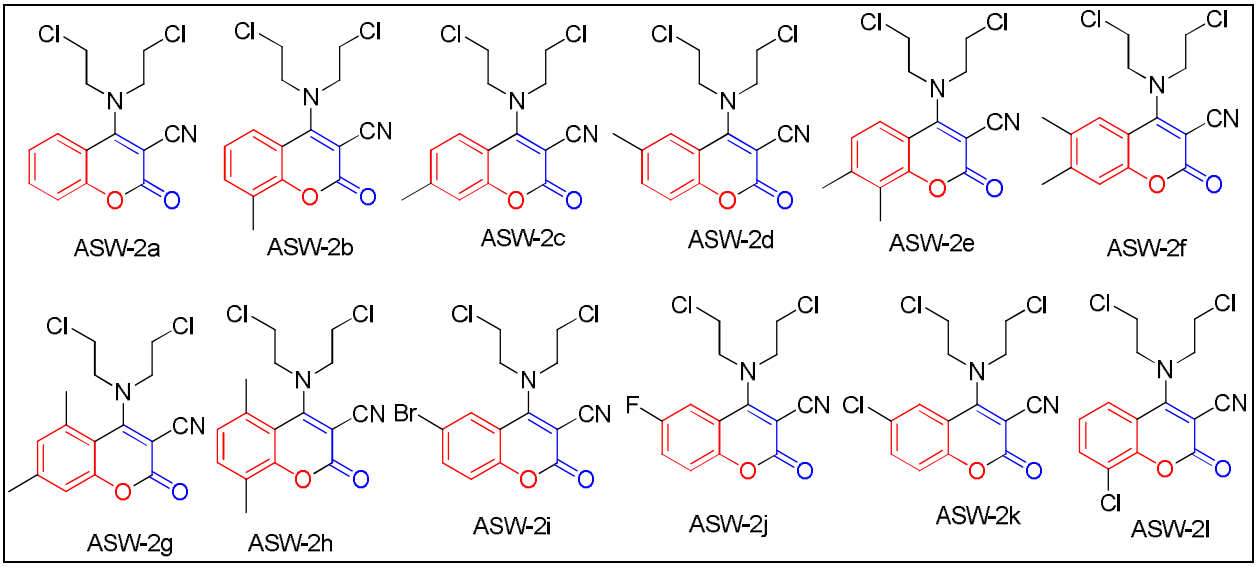
Scheme 1;(a) $POCl_3$, $AnhyZnCl_2$, 80°C, 5-6 hr **(b)** DMF, $POCl_3$, 0-60°C **(c)** NH_2OH, $Gly.CH_3COOH$, CH_3COONa **(d)** IPA, excess ethanol amine **(e)**DMF, $SOCl_2$, 0°C to rt.

Table 1: Physical property of synthesized coumarin based N-nitrogen mustard

Code	Molecular Formula	R	Molecular Weight	Melting Point °C	Yield %
Asw2a	$C_{14}H_{12}Cl_2N_2O_2$	H	310	184	73
Asw2b	$C_{15}H_{14}Cl_2N_2O_2$	2-CH_3	324	186	68
Asw2c	$C_{15}H_{14}Cl_2N_2O_2$	3-CH_3	324	148	63

Asw2d	$C_{15}H_{14}Cl_2N_2O_2$	4-CH_3	324	208	66
Asw2e	$C_{16}H_{16}Cl_2N_2O_2$	2,3-diCH_3	338	174	69
Asw2f	$C_{16}H_{16}Cl_2N_2O_2$	3,4-diCH_3	338	178	78
Asw2g	$C_{16}H_{16}Cl_2N_2O_2$	3,5-diCH_3	338	194	67
Asw2h	$C_{16}H_{16}Cl_2N_2O_2$	2,5-diCH_3	338	190	55
Asw2i	$C_{14}H_{11}BrCl_2N_2O_2$	4-Br	387	196	64
Asw2j	$C_{14}H_{11}Cl_2FN_2O_2$	4-F	328	216	61
Asw2k	$C_{14}H_{11}Cl_3N_2O_2$	4-Cl	343	220	63
Asw2l	$C_{14}H_{11}Cl_3N_2O_2$	2-Cl	343	222	68

Scope of substrate



Spectral data of the synthesized compounds

4-(bis(2-chloroethyl)amino)-2-oxo-2H-chromene-3-carbonitrile (ASW-2a);Brown solid; R_f0.41 (8:2 EA-hexane); mp 190°C; IR (KBr, cm^{-1}): 3278, 1680, 840, 752, 690, 623 cm^{-1}; ^{1}H NMR: δ $_{PPM}$ 8.62 to 8.35 (m, 1H, Ar-H), 7.74(tri, 1H, Ar-H), 6.783(s, 2H, Ar-H),3.749(s, 8H, $(-CH_2CH_2-)_2$). ^{13}C NMR (400 MHz, DMSO): 41.05, 52.09, 111.61, 114.50, 124.55, 125.14, 126.74, 134.05, 145.18, 151.30, 155.38.MS (*m/z*): 310 (M^+).

4-(bis(2-chloroethyl)amino)-8-methyl-2-oxo-2H-chromene-3-carbonitrile (ASW-2b): Brown solid; R_f0.39 (8:2 MDC-hexane); mp 181-183°C; IR (KBr, cm^{-1}): 3298, 1681, 786, 723, 645 cm^{-1}; MS (*m/z*): 324 (M^+).

4-(bis(2-chloroethyl)amino)-7-methyl-2-oxo-2H-chromene-3-carbonitrile(ASW-2c) Brown solid; R_f0.45 (8:2 EA-hexane); mp 146-148°C; IR (KBr, cm^{-1}): 3294, 1680, 866, 821, 786, 723, 644 cm^{-1}; MS (*m/z*): 324 (M^+).

Biological activity

Principle :Anticancer sensitivity testing

The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 96-wellformat. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloro acetic acid and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mMTris base solution for OD determination at 510 nm using a micro platereader. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods. The method not only allows a largenumber of samples to be tested within a few days, but also requires only simple equipment and inexpensive reagents. The SRB assay is therefore an efficient and highly cost-effective method for screening.

	Drug concentrations (µg/ml) calculated from graph		
PC-3	**LC_{50}**	**TGI**	**GI_{50}**
ASW-2a	>100	>100	>100
ASW-2b	>100	>100	>100
ASW-2c	>100	>100	>100
ASW-2d	>100	>100	98.26102
ASW-2e	>100	>100	97.2
ASW-2f	>100	>100	87.2
ASW-2g	>100	>100	90.4
ADR	61.2	19.1	<10

Cytotoxicity Data of synthesized compounds

	Human Prostate Cancer Cell Line PC3															
	% Control Growth															
	Drug Concentrations (µg/ml)															
Compound code	**Experiment 1**				**Experiment 2**				**Experiment 3**				**Average Values**			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**	**10**	**20**	**40**	**80**
ASW-2a	100.0	100.0	100.0	57.8	100.0	100.0	100.0	63.5	100.0	100.0	96.3	61.1	100.0	100.0	98.8	60.8
ASW-2b	100.0	100.0	100.0	64.3	100.0	100.0	97.4	66.1	100.0	100.0	95.4	72.9	100.0	100.0	97.6	67.8
ASW-2c	100.0	100.0	98.0	60.4	100.0	100.0	90.5	57.7	100.0	100.0	87.3	77.1	100.0	100.0	91.9	65.1
ASW-2d	100.0	100.0	99.6	52.7	100.0	100.0	97.0	53.5	100.0	100.0	90.6	54.2	100.0	100.0	95.7	53.5
ASW-2e	100.0	100.0	100.0	52.5	100.0	100.0	95.2	47.7	100.0	100.0	100.0	55.5	100.0	100.0	98.4	51.9
ASW-2f	100.0	100.0	100.0	39.9	100.0	100.0	100.0	43.3	100.0	100.0	100.0	50.5	100.0	100.0	100.0	44.6
ASW-2g	100.0	100.0	100.0	40.7	100.0	100.0	100.0	50.1	100.0	100.0	100.0	50.1	100.0	100.0	100.0	47.0
ADR	-30.8	-42.4	-44.9	-48.4	-31.3	-39.3	-45.5	-46.3	-42.9	-43.2	-46.7	-50.1	-35.0	-41.7	-45.7	-48.3

Adriamycin is taken as standard for this experiment

Conclusion

We have serve number of novel nitrogen mustard containing functionalized coumarin as potent antitumor agents using novel synthetic approaches in high yield and purity. The reaction of diethanol amine and various 4-chloro 3-cyano coumarins was carried out by simply in *IPA* and excess diethanol amine as a base. Latter on chlorination of 4-(bis(2-hydroxyethyl)amino)-2-oxo-2H-chromene-3-carbonitrile result in 4-(bis(2-chloroethyl)amino)-2-oxo-2H-chromene-3-carbonitrile derivatives. The formation of coumarin *N*-mustards by this method was first developed by us. All the synthesized compounds were evaluated for their anticancer activity. The investigation of anticancer screening data revealed that the compounds show moderate activity at higher concentration. All synthesized compounds were obtained in good to moderate yield. All synthesized compounds were characterized by IR, NMR and Mass spectrometry.

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