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Anti-tumor agents: Design, Synthesis, and Biological study of *N*-Substituted-7-hydroxy-1-azacoumarin-3-carboxamide derivatives as potent cytotoxic agents

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Synthesis of ethyl 7-hydroxy-1-azacoumarin-3-carboxylate (**3**) was developed using ethyl-7-hydroxy coumarin-3-carboxylate and ammonium solution as the key synthons. Condensation of ethyl 7-hydroxy-1-azacoumarin-3-carboxylate with ammonium acetate and aniline to give *N*-substituted-7-hydroxy-1-azacoumarin-3-carboxamides (7-Hydroxy -1-azacoumarin-3-carboxamide (**4**) and *N*-phenyl 7-Hydroxy-1-azacoumarin-3-carboxamide (**5**)). Bromo derivative (*N*-phenyl 6, 8-dibromo-7-hydroxy-1-azacoumarin-3-carboxamide (**6**)) was obtained from halogenation of compound *N*-phenyl 7-Hydroxy-1-azacoumarin-3-carboxamide (**5**) with bromine in glacial acetic acid. *N*-phenyl-2,5-diacetoxy-6, 8-disubstituted-Quinoline-3-carboxamides (*N*-phenyl 2,7-diacetoxy-Quinoline-3-carboxamide (**7**) and *N*-phenyl 2,7-diacetoxy-6,8-dibromo-Quinoline-3-carboxamide (**8**)) were prepared via the acetylation of compounds 5 and 6 with acetic anhydride. Five compounds **4–8** were evaluated *in vitro* against more than one human tumor cell lines. Among the selected compounds, **6** showed the best *in vitro* cytotoxicity against the human cancer cell line; MCF-7 (with IC_{50} = 10.12 μM). In addition, cell cycle analysis of compound **6** demonstrated cell cycle arrest at G2/M phase and Pre-G1 apoptosis.

Keywords: azacoumarin-3-carboxamide; cytotoxicity; ^{1}H, ^{13}C-NMR spectra; MCF-7.

INTRODUCTION

Coumarins and their derivatives are widely present in higher plants such as Thymelaeaceae, Rutaceae, Leguminosae, Asteracease, Apiaceae, as well as occur as animal and microbial metabolites[1]. Coumarin derivatives play an important wide spectrum of pharmacological effects including anti-arrhythmic, antiosteoporosis, anti-HIV, antimicrobial, and anticancer activities[2–5]. We became interested in the less commonly studied 7-hydroxy-2(1H) quinolines, the aza analogues of 7-hydroxy Coumarins. Biological evaluation of hydroxyl quinolones revealed antitumor activity for the chloro derivatives[6] as well as antitumor activity for some 2-phenyl substituted -3-hydroxy-4(1H)-Quinolinone-7-carboxlic acids phenacyl esters[7]. In addition, inhibition of DNA gyrase and topoisomerase II by hydroxyl quinolinones has been reported by Sui et al.[8]. The promising pharmacology properties of 3-hydroxy-4-Quinolinones (cytotoxicity to cell lines As4s, K562, K562-Tax, CEM-DNRB) prompted us to carry out a detailed study of the structure-activity relationship[9].

Herein, we state the synthesis of some 7-hydroxy -2-oxo--quinoline-3-carboxamide hybrids to achieve improved antitumor activity comparison with 3-hydroxy-4-(1*H*) Quinolinones, the aza analogues of hydroxyl Coumarins.

This paper reported the use of a simple method in the preparation of 7-hydroxy-1-azacoumarins from the analoges 7-hydroxy coumarin derivative, cytotoxicity against different human tumor cell lines, and preliminary pharmacological mechanism of new compounds.

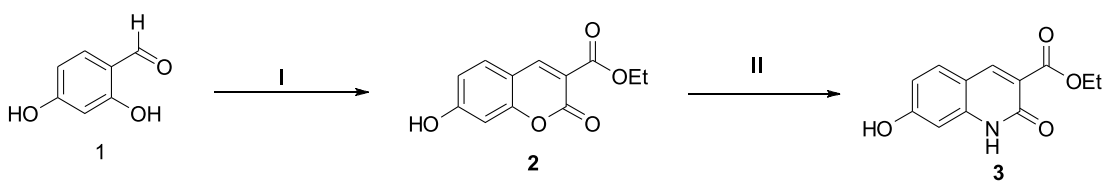
DESIGN

Therefore, five *N*-Substituted 7-hydroxy-1-azacoumarin--3-carboxamide derivatives were designed, synthesized, screened for cytotoxic activity, and evaluated for the effect of diverse substituents on the potency. Based on the promising degree of cytotoxicity shown by certain brominated *N*-substituted 7-hydroxy-1-azacoumarin-3-carboxamides. All synthesized compounds were screened *in vitro* against two different human cancer cell lines. Compound (**6**) with the best activity was tested independently in cell proliferation, apoptosis and cell cycle assays in the human breast cancer cell line.

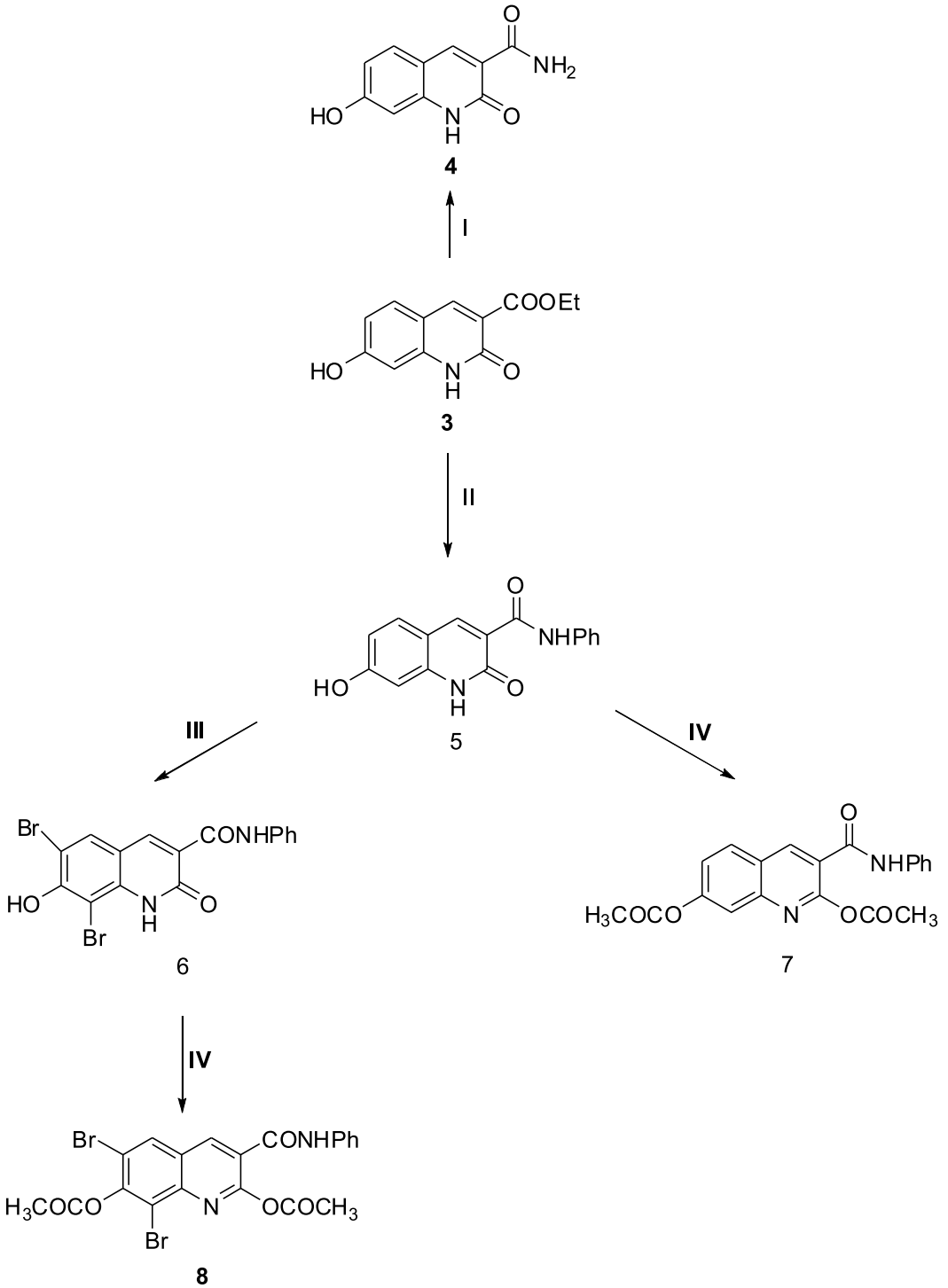
RESULTS AND DISCUSSION

Chemistry

As shown in **Scheme 1**, commercially available 2,4-dihydroxy benzaldehyde (**1**) was reacted with diethyl malonate in piperidine as catalyst under fusion to form ethyl 7-hydroxy coumarin-3-carboxylate (**2**), which was subsequently converted to ethyl 7-hydroxy-1-azacoumarin-3-carboxylate (**3**) with ammonia solution in ethanol in the presence of anhydrous potassium carbonate under reflux. The synthesis of *N*-Substituted-1-azacoumarin-3-carboxamide



Scheme 1. Synthesis of ethyl 7-hydroxy-1-azacoumarin-3-carboxylate (**3**)
Reaction conditions: **I**) diethyl malonate/ piperidine, **II**) EtOH/ K_2CO_3/ NH_3/ reflux



Scheme 2. Synthesis of N-Substituted-1-azacoumarin 3-carboxamide derivatives (**4–8**).
Reaction conditions: **I**) Ammonium acetate/ fusion, **II**) aniline/ ACOH, **III**) Bromine/ ACOH, **IV**) acetic anhydride

derivatives (**4–8**) is described in **Scheme 2**. Treatment of ethyl 7-hydroxy-1-azacoumarin-3-carboxylate (**3**) with ammonia furnished from ammonium acetate under fusion led to the formation of 7-hydroxy-1-azacoumarin-3-carboxamide (**4**). Also, *N*-phenyl-7-hydroxy-1-azacoumarin--3-carboxamide (**5**) was obtained by the condensation of ester 1-azacoumarin (**3**) with aniline in acetic acid under reflux. The reaction of *N*-phenyl 7-hydroxy-1-azacoumarin -3-carboxamide (**4**) with bromine in glacial acetic acid with stirring at 60°C afforded to *N*-phenyl-6,8-dibromo-7-hydroxy-1-azacoumarin-3-carboxamide (**6**).

The structures of compounds **5** and **6** were confirmed *via* its transformation into *N*-phenyl-7-acetoxy-2-acetoxy-quinoline-3-carboxamide (**7**) and *N*-phenyl-6, 8-dibromo-7-acetoxy-2-acetoxy-quinoline-3-carboxamide (**8**), respectively *via* acetylation of compounds **5** and **6** with acetic anhydride under reflux. All the new synthesized compounds have been described by IR, ^{1}HNMR, ^{13}CNMR and elemental analysis. From the study, the NMR data of compounds **4, 7** and **8** showed the structure of these compounds into DMSO-d_6 solutions in keto-enoltautomers as shown in chart-1. The signals of keto form and enol form of these compounds are listed in the experimental section.

Evaluation of biological activity

In vitro cytotoxic activity against MCF-7 cell line

Cell proliferation was measured by MTT assay, which measures the ability of the cells to convert tetrazolium

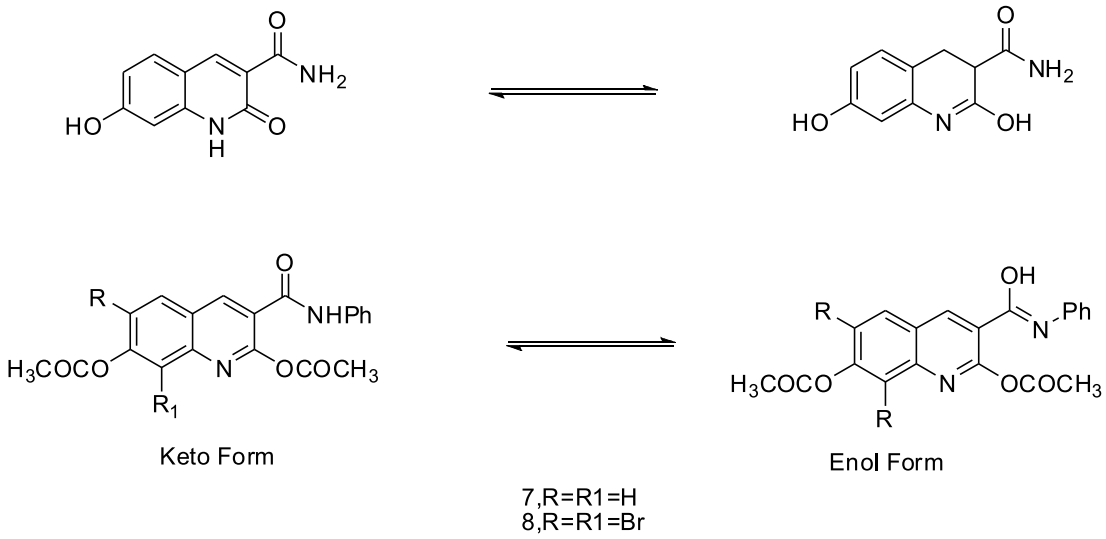


Chart 1. Keto-enoltautomers for compounds **7** and **8**

salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) a yellow tetrazole to the purple formazan product. Doxorubicin (Dox) was used as a reference drug in this study. It was observed that compound **6** and its acetylated derivative **8** demonstrated the most potent cytotoxic activity with IC_{50} values of 10.12 and 14.62 μM, respectively.

Table 1. *In vitro* antitumor activity of *N*-Substituted-7-hydroxy--1-azacoumarin-3-carboxamide **4–8** compared with Dox

Compound No.	IC_{50} values (μM)
	MCF-7
4	19.21±0.93
5	15.21±0.21
6	10.12±0.22
7	>50
8	14.62±0.63
Dox	3.17±0.06

Cell cycle analysis

The most cytotoxic compound **6** was selected to further evaluate its effect on the cell cycle distribution in MCF-7 cell line. Treatment of MCF-7 cells to compound **6** at its IC_{50}concentation dose value for 24 followed by DNA flow cytometry assay were analyzed[10]. As illustrated in (**Figure 1**) compound **6** induces an increase in the percentage of the cells at G2/M phase by 1.74 – compared to untreated control. In conclusion, compound **6** could both result in the arrest MCF-7cells in G2/M phases.

Annexin V-FITC/PI and detection of apoptosis of compound 6

To determine the apoptosis-inducing activity of compound **6**[11]**.** A biparametric cytofluorimetric analysis was performed for compound **6** at its IC_{50} concentration, using AnnexinV/Propidium iodide (PI). As shown in (**Figure 2**), the selected compound showed a sharp decrease in the cell survival percentage in comparison with the control. Also, compound **6** increase the percentage of early apoptotic cells by 7.85 – fold more than control. Moreover, compound **6** increases the percentage of late apoptotic cells by 18.07 – more than control. The results suggested that compound **6** increase the apoptosis of MCF-7 cells.

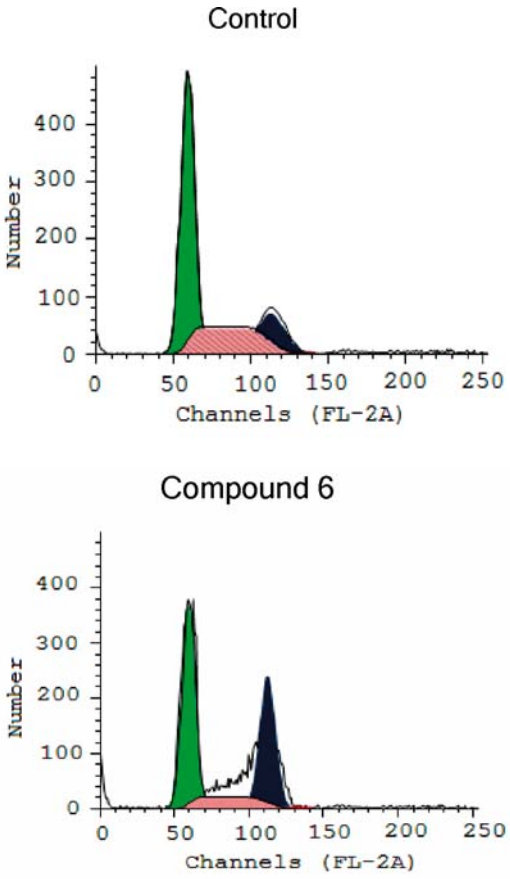


Figure 1. Cell cycle analysis of compound **6** compared to untreated control

CONCLUSION

Five novel *N*-Substituted-7-hydroxy-1-azacoumarin--3-carboxamide (**4, 5** and **6**) and *N*-phenyl substituted 2,7-diacetoxy-quinoline-3-carboxamides (**7** and **8**) were designed and synthesized from commercially available 2,4-dihydroxy benzaldehyde as a key starting material. The structures of compounds **3–8** were confirmed by IR, ^{1}H, ^{13}C-NMR and elemental analysis. The prepared compounds **4–8** were assessed for their *in vitro* antiproliferative activity against MCF-7 cancer cell line. Cell cycle analysis of compound **6** demonstrated cell cycle arrest at G2/M phase and Pre-G1 apoptosis.

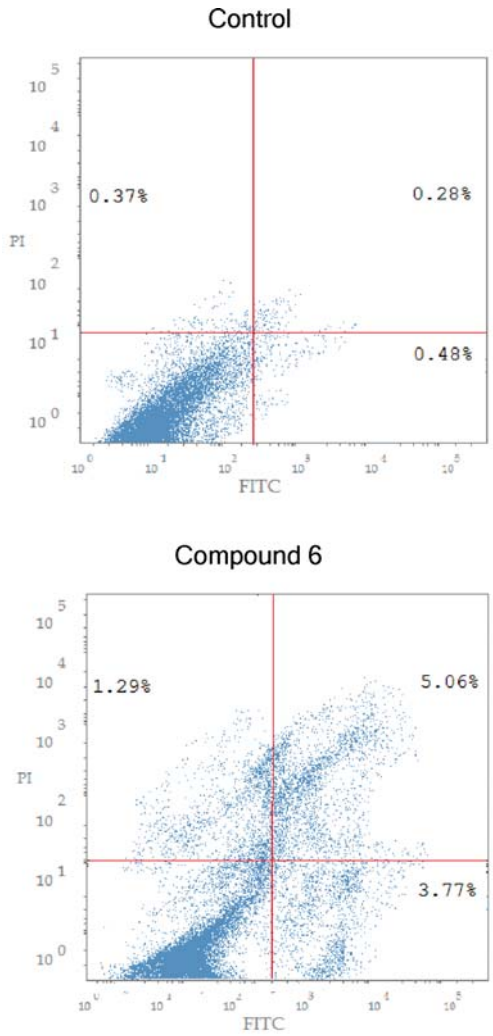


Figure 2. Annexin V-FITC/PI analysis of compound **6** compared to untreated control

EXPERIMENTAL

Instrumental analysis

Melting points were measured with an Electrothermal IA9200 Programmable Digital Melting Point Apparatus. The 1H, 13C-NMR spectra were measured on Bruker 400 MHz Avance III nuclear magnetic resonance (NMR) spectrometer using TMS as the internal standard. Mass spectra were resolved by Electron Ionization (EI) Mass Spectrometry using an Agilent 5975C Series GC/MSD Spectrometer operating at 70 eV. The elemental analysis was performed on a PerkinElmer® 2400 Series II CHN-S/O Elemental Analyzer. Chemical and reagents were purchased from Sigma-Aldrich Chemical Company. All reagents were of analytical grade.

Synthesis

Ethyl 7-hydroxy coumarin-3-carboxylate (2)

A mixture of the equimolar quantity of 2, 4-dihydroxy benzaldehyde (0.01 mol) and diethyl malonate (0.01 mol) in the presence of piperidine was fused on a hot plate for 2–3 min. The reaction mixture was added ethanol (50 mL) then heating under reflux for 2 h, then cooled down and poured into water. The reaction mixture was neutralized with a few drops from hydrochloric acid (4N), The solid formed was collected by filtration, washed with water, dried, and purified with crystallization from the ethanol to give **2**.

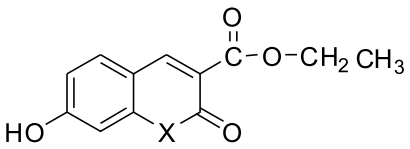
As colorless crystals, yield 78%, m.p. 187°C. IR (KBr) υ_{max}: 3435(br.OH), 1763, 1728(C=O), 1617, 1589(C=C), 1125, 1083(C-O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.32(t, 3H, CH_3), 4.22(q, 2H, OCH_2), 6.67(s, 1H, H-8 of coumarin ring), 6.78–6.80(d, 1H, H-6 of coumarin ring), 7.67–7.69(d, 1H, H-5 of coumarin ring), 8.61(s, 1H, H-4 of coumarin ring), 11.08(br.s, 1H, OH) ppm.^{13}C-NMR(DMSO-d_6) δ: 164.51(C-11), 163.33(C-2), 157.56(C-7), 156.77(C-9), 149.78(C-4), 132(C-10), 114.44(C-5), 112.08(C-6), 110.81(C-8), 102.19(C-3), 61.23(C-13), 14.54(C-14)ppm.

Ethyl 7-hydroxy-1-azacoumarin-3-carboxylate (3)

A mixture of compound **2** (0.01 mol) and anhydrous potassium carbonate (0.03 mol) in ethanol was refluxed for 30 min. Then ammonia solution (10 mL, 35%) was added to the reaction, followed by heating under reflux 2 h, then cooled down and poured into water. The reaction mixture was neutralized with dilute hydrochloric acid (4N), The precipitate formed was separated, washed with water, and dried. Finally, the resulting product was crystallized with ethanol to give **3a**.

As pale yellow crystals, yield 71%, m.p. 130°C; IR (KBr) υ_{max}: 3431 (OH), 3263(NH), 1738, 1681(C=O), 1607, 1583(C=C), 1121, 1093, 1027(C-O) cm^{-1}.

^{1}H-NMR (DMSO-d_6) δ: 1.32(t, 3H, CH_3), 4.25(q, 2H, OCH_2), 6.72(S, 1H, H-8 of azacoumarin ring), 6.85(d, 1H, H-6 of azacoumarin ring), 7.75(d, 1H, H-5 of azacoumarin ring), 9.71(br.s, 1H, OH) ppm.^{13}C-NMR(DMSO-d_6) δ: 164.67(C-11), 163.41(C-2), 157.59(C-7), 156.88(C-9), 149.89(C-4), 132.32(C-5), 114.52(C-10), 112.44(C-6), 110.83(C-8), 102.26(C-3), 61.27(C-13), 14.60(C-14) ppm. MS: m/z(%) = 233(M+, 16.3). Anal. Calcd for $C_{12}H_{11}NO_4$(233): C, 61.80; H, 4.72; N, 6.01. Found: C, 61.66; H, 4.44; N, 5.83.



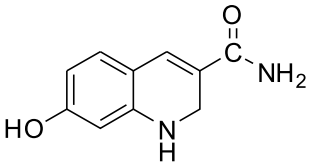
2; X = O, **3;** X = NH

7-Hydroxy -1-azacoumarin-3-carboxamide (4)

A mixture of ethyl 7-hydroxy -1-azacoumarin-3-carboxylate (3, 0.01 mol) and ammonium acetate (0.05 mol) was fused on a hot-plate at 100–110°C for 2 h, then cooled down and poured into the water with stirring. The resulting product was filtered off, washed with water, dried, and recrystallized from ethanol to give **4**.

As red color crystals, yield 61%, and m.p. 205°C; IR (KBr) υ_{max}: 3452(br-OH), 2305, 3167(NH_2), 1705-1690(br.C=O), 1605, 1592(C=C), 1095, 1036(C-O) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 6.73(s, 1H, H-8 of azacoumarin ring), 6.81–6.82(d, 1H, H-6 of azacoumarin ring), 7.72–7.74(d, 1H, H-5 of azacoumarin ring), 7.97(br.s, NH_2), 8.72(s, 1H, H-4 of azacoumarin ring), 11.02(br.s, 1H, OH)ppm.^{13}C-NMR (DMSO-d6) δ: 164.11(C-2), 163.49(C-11), 161.46(C-7), 156.93(C-9), 148.91(C-4), 132.48(C-5), 114.75(C-10), 114.39(C-6), 111.58(C-5), 102.25(C-3) ppm. MS (m/z, %) = 204(M^+, 11.23). Anal.

Calcd for $C_{10}H_8N_2O_3$ (204): C, 58.82; H, 3.92;N, 13.73. Found: C, 58.58; H, 3.67; N, 13.47.

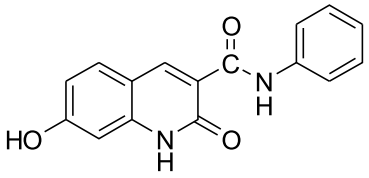


4

N-phenyl 7-Hydroxy-1-azacoumarin-3-carboxamide (5)

A solution of compound 3 (0.01 mol) in acetic acid (25 mL) was added aniline (0.01 mol) and heating under reflux for 4 h, then cooled down and poured into water with stirring. The resulting product was separated by filtration, washed with water, dried and recrystallized with ethanol to give **5**.

As yellow crystals, yield 67%, and m.p. 221°C; IR (KBr) υ_{max}: 3452(br.OH), 3223(NH), 1710-1701(br. C=O), 1612, 1583(C=C), 1172, 1093, 1036(C-O) cm^{-1}.^{1}H-NMR(DMSO-d_6) δ: 6.77(s, 1H, H-8 of azacoumarin), 6.82–6.85(d, 1H, H-6 of azacoumarin ring), 7.01–7.07(t, 2H, Ar-H), 7.28–7.32(t, 2H, Ar-H), 7.63–7.64(t, 1H, Ar-H), 7.93(d, 1H, H-5 of azacoumarin), 8.80(s, 1H,H-4 of azacoumarin), 10.58(s, 1H, NHCO), 11.095(br.s, N=C-OH)ppm.^{13}C-NMR (DMSO-d_6) δ: 164.43(C-11), 161.81(C-2), 160.64(C-7), 156.85(C-9), 148.87(C-13), 138.51(C-4), 132.64(C-5), 129.48(C-15,17), 124.59(C-16), 120.29(C-14,16), 115.02(C-10), 114.53(C-6), 111.71(C-8), 102.37(C-3) ppm. MS: m/z (%) = 280(M^+, 13.62), Anal. Calcd for $C_{16}H_{12}N_2O_3$ (280): C, 68.57; H, 4.29; N, 10.00. Found: C, 68.35; H, 4.09; N, 9.82.

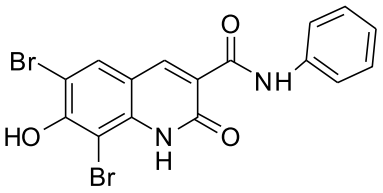


5

N-phenyl 6, 8-dibromo-7-hydroxy-1-azacoumarin-3-carboxamide (6)

In 25 mL of glacial acetic acid, *N*-phenyl 7-hydroxy-1-azacoumarin-3-carboxamide (**5**, 0.01 mol) was dissolved. Then 10 mL of bromine (0.02 mol) in glacial acetic acid was added dropwise to compound **5** solution with stirring at 60°C. After 5–10 min the bromine color was discharged and a yellow solution remained. At this point, 0.5–1 mL of bromine–ACOH solution was added with stirring at room temperature for 1h. The reaction mixture was poured into water with stirring, and the solid formed was collected by filtration, washed with water, and dried. Finally, the product was recrystallized from ethanol to give **5**.

As orange crystals, yield 78%, m.p. 185°C; IR (KBr) υ_{max}: 3512 (br.OH), 3287(NH), 1718, 1705(C=O), 1613, 1589(C=C), 1091, 1034(C-O)cm^{-1}. ^{1}H-NMR(DMSO-d_6) δ: 7.55–8.44 (m, 6H, Ar-H and H-5 of azacoumarin ring), 8.80(s, 1H, H-4 of azacoumarin; keto form), 8.94(s, 1H, H-4 of azacoumarin; enol form), 10.60(s, 1H, NH, CONH, keto-form), 11.07(s, 1H, OH, N=C-OH, enol-form)ppm.^{13}C-NMR (DMSO-d_6) δ: 160.42(C-11 of keto-form), 157.01(C-H, enol form), 159.30(C-2), 149.31(C-7), 147.38(C-9), 143.30,137.75(C-13), 133.99, 133.36(C-4), 133.02(C-5), 132.28(15,17), 131.80(C-16), 122.36(C-14,18), 117.00(C-10), 114.66, 113.75(C-8), 108.78, 108.42(C-6), 99.27(C-3) ppm. MS: m/z (%) = 436 (M^+, 10.31). Anal. Calcd. for $C_{16}H_{10}N_2Br_2O_3$ (436): C, 44.04; H, 2.29; N, 6.42. Found: C, 43.84; H, 2.08; N, 6.22.



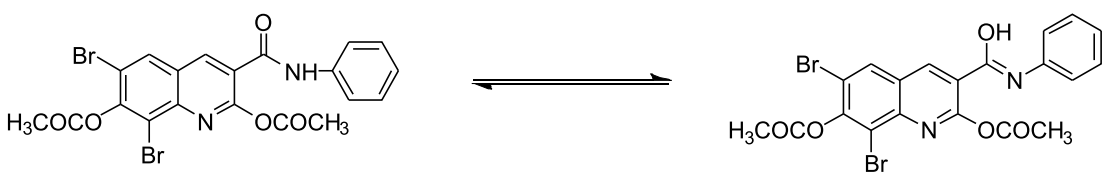
6

N-phenyl 2,7-diacetoxy-6,8-disubstituted-Quinoline-3-carboxamides (7 and 8).

A solution of compounds **5** and **6** (0.01 mol) in acetic anhydride (20 mL) was heated under reflux 2 h, then cooled down and decanted into water. The reaction mixture was left 24 h, and the solid formed was separated by filtration, washed with water, and dried. Finally, the product was recrystallized from a suitable solvent to give **7** and **8**.

N-phenyl 2,7-diacetoxy-Quinoline-3-carboxamide (7)

Yield 61%, m.p. 145°C; IR (KBr): 3265(NH), 1766, 1695(C=O), 1632(C=N), 1605, 1583(C=C), 1135, 1092, 1032(C-O) cm^{-1}. ^{1}H-NMR(DMSO-d6) enol-keto (Chart-2) form δ: 1.99(s, 3H, CH_3), 2.34(s, 3H, CH_3), 7.18(t, 1H, Ar-H), 7.24–7.31(d, 1H, Ar-H), 7.38–7.40(t, 2H, Ar-H), 7.46–7.56(m, 3H, Ar-H and H-8 of azacoumarin), 7.72–7.74(d, 1H, H-6 of azacoumarin), 7.89–7.91(d, 1H, H-5 of azacoumarin), 8.38(s, 1H, H-4 of Quinoline), 10.63(s, 1H, NH, NHCO) ppm. ^{1}H-NMR(DMSO-d6) enol-form δ: 1.92(s, 3H, CH_3), 2.33(s, 3H, CH_3), 7.18(t, 1H, Ar-H), 7.24–7.31(d, 1H, Ar-H), 7.38–7.40(t, 2H, Ar-H, Ar-H), 7.46–7.56(m, 3H, Ar-H and H-8 of Quinoline), 7.72–7.74(d,1H, H-6 of Quinoline), 8.06–8.08(d, 1H, H-5 of Quinoline), 9.39(s, 1H, H-4 of Quinoline), 12.09(br.s, 1H, OH, N=C-OH) ppm.^{13}C-NMR(DMSO-d_6) of keto-form δ: 172.11(C-2), 169.17(C-23), 166.87(C-20), 160.28(C-11),



Keto-form Enol-form

Chart 2. Enol-Keto form of compound **7**

155.14(C-7), 154.43(C-9), 142.13(C-13), 138.38(C-4), 131.80(C-5), 130.21(C-10), 129.53(C-15,17), 125.51(C-16), 120.38(C-14,18), 119.88(C-8), 116.87(C-6), 110.69(C-3), 27.18(C-24), 21.53(C-21) ppm.^{13}C-NMR(DMSO-d_6) of enol-form δ: 173.17(C-2), 169.23(C-23), 160.82(C-20), 157.74(C-7), 155.01(C-11), 154.32(C-9), 147.50(C-13), 138.81(C-4), 130.80(C-5), 130.21(C-10), 129.30(C-15,17), 124.82(C-16), 120.09(C-14,18), 119.66(C-8), 116.47(C-6), 110.53(C-3), 25.66(C-24), 21.36(C-21) ppm. Anal. Calcd for $C_{20}H_{16}N_2O_5$(364): C, 65.93; H, 4.40; N, 7.69. Found: C, 65.65; H, 4.22; N, 7.47.

N-phenyl 2,7-diacetoxy-6,8-dibromo-Quinoline-3-carboxamide (8)

Yield 63%, m.p. 141°C. IR(KBr) υ_{max}: 3221(br NH), 1768, 1695(C=O), 1633(C=N), 1609, 1591(C=C), 1117, 1083, 1021(C-O) cm^{-1}. ^{1}H-NMR (DMSO-d6) of keto-form (Chart 3) δ: 2.40(s, 3H, $COCH_3$), 2.66(s, 3H, $COCH_3$), 7.40–8.32(m, 5H, Ar-H), 8.46(s, 1H, H-5 of Quinoline), 8.92(s, 1H, H-4 of Quinoline), 10.92(br.s, 1H, OH, N=C-OH) ppm. ^{13}C-NMR (DMSO-d_6) of keto-form δ: 167.49(C-2), 162.81(C-23), 159.93(C-20), 159.66(C-11), 159.36(C-7), 151.40(C-9), 146.00(C-13), 135.75(C-4), 135.02(C-5), 132.86(C-10), 122.40(C-15,17), 122.28(C-16), 119.49(C-8), 117.24(C-6), 114.98(C-3), 113.21(C-14,18), 20.71(C-21), 31.25(C-24) ppm. ^{13}C-NMR (DMSO-d_6) of enol-form δ: 175.55(C-2), 163.56(C-23), 160.20(C-20), 159.52(C-7), 151.53(C-11), 150.15(C-9), 149.78(C-13), 137.58(C-4), 133.24(C-5), 132.30(C-10), 123.70(C-15,17), 121.70(C-16), 119.40(C-8), 116.69(C-6), 114.77(C-3), 111.02(C-14,18), 21.55(C-21), 34.86 (C-24) ppm. Anal. calcd for $C_{20}H_{14}N_2Br_2O_5$ (520): C, 46.15; H, 2.69; N, 5.38. Found: C, 46.01; H, 2.36; N, 5.15.

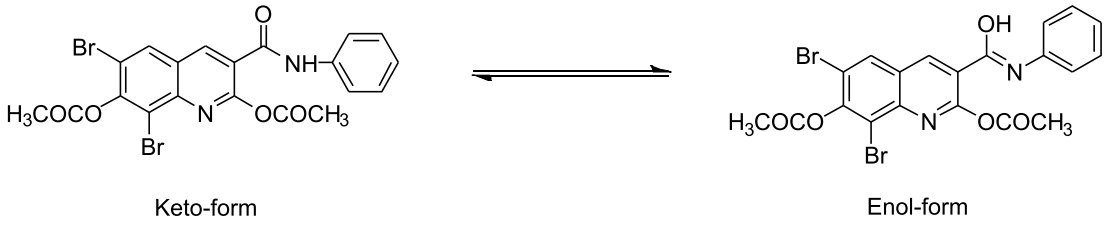


Chart 3. Enol-Keto form of compound **8**

Biological Evaluation

Anti-tumor activity against breast cancer cell line (MCF-7)

The cytotoxic activity was measured *in vitro* for the prepared derivatives using the MTT assay. Cells were plated in 96-multiwall plate (10^5 cells/well) for 24 h before treatment with the compounds. Test compounds were dissolved in dimethyl sulfoxide. Different concentrations of the compound under test (10, 25, 50, and 100 μM) were added to the cell's monolayer. Triplicate wells were prepared for each concentration. Monolayer cells were incubated with the compounds for 48 h at 37°C and in the atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained with 40μL of MTT solution (5 mg/mL of MTT in 0.9% NaCl) in each well was added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 μL of acidified isopropanol/well and the plate was shaken at room temperature, followed by photometric determination of the absorbance at 570 nm using ELISA reader. The molar concentration required to inhibit 50% of cell viability (IC_{50}) was calculated and compared with the reference drug doxorubicin. The surviving fractions were expressed as means ± S.E.M.

Cell cycle analysis of compound 6

MCF-7 cells, (3.0 x 10^5 cells/well) and incubated at 37°C for 12 h. The target cells were then treated with the compound **6** at its IC_{50} concentration dose value for 24 h. After treatment, cells were collected and fixed with 75% ethanol at 20°C overnight, then, cells were washed with PBS followed by centrifugation and incubated with (10 mg/mL) RNase (Sigma, USA) and (5 mg/mL) propidium iodide (PI, Sigma) before flow cytometry analysis (*FACSCalibur* cytometer using Cellquest software, BD Bioscience, USA).

Apoptosis determination by Annexin-Assay

The MCF-7 cells (2 x 10^5/ well) were treated with compound**6**at its IC_{50} concentration value for 24 h. After treatment, cells were harvested and washed twice (180 g, 10 min, 4°C) with PBS. Each cell well was resuspended in 100 µL of binding buffer, and 5 μL Annexin V-FITC was added. After an incubation time of 10 min at room temperature, an additional 400 μL of binding buffer was added for a final volume of 500 μL. Cells were stained with PI immediately before measurement. Cells were analyzed by using *FACSCalibur* Flow cytometer (Becton and Dickinson, Heidelberg, Germany). Data thus obtained were analyzed with Cell-Quest software (Becton and Dickinson, Heidelberg, Germany).

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