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Synthesis and Urease Enzyme Inhibitory Effects of Some Dicoumarols

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Dicoumarols 1–10 with substituted phenyl residues at C-11 were synthesized and screened for their urease inhibition effects. All synthesized compounds showed varying degree of urease inhibitory activity ranging from IC_{50} = 74.30–91.35 μM.

Keywords: 4-Hydroxycoumarin; Dicoumarol; Derivatives; Urease Inhibitors

INTRODUCTION

Coumarins posses a wide range of biological activities, occupying a special place in the realm of natural and synthetic organic chemistry. Diverse biological activities for compounds incorporating this ring system, including molluscicid,[1] anthelmintic, hypnotic, insecticidal,[2] and anticoagulant[3] are reported in literature. Some of the coumarin derivatives are fluorescent brighteners[4] and are used as a precursor for the synthesis of other natural products, such as furocoumarins, chromones, coumarones and 2-acylresorcinol and dicoumarol.[5] Dicoumarols show interesting anticoagulant activity and were also identified as haemorrhagic agent in the spoiled clover disease of cattle.[6] Such compounds can easily be synthesized by condensing formaldehyde with two equivalent of 4-hydroxycoumarin.[6] In nature, dicoumarol is found in *moldy clover*,[7] and one of its derivatives, gerberinol has been isolated from *Gerbera lanuginosa* (Benth),[8] and from many other plants, especially from the family *Compositae*.[9]

Two types of dicoumarols, symmetrical and unsymmetrical are reported in the literature.[10] Wolff prepared dicoumarol by condensing 4-hydroxycoumarin with one equivalent of aldehyde or ketone.[11] Anschutz reported the condensation of 4-hydroxycoumarin with formaldehyde and acetaldehyde in aqueous solution, but was unable to condense it with propionaldehyde, butyraldehyde or acetone under similar conditions.[12] Sullivan *et al.*, reported the synthesis of dicoumarol by refluxing 4-hydroxycoumarin with various aldehydes in ethanol, but in poor yields.[13] Abramovitch *et. al.* first synthesized 4-hydroxy-7-methyl-3-*N*-piperidinomethylcoumarin from dicoumarol through a long reaction procedure by refluxing aldehyde and 4-hydroxycoumarin in ethanol using piperidine as base.[10]

Recently we explored a variety of classes of compounds in our drug discovery program.[14–20] Taking into account the diverse biological activities of dicoumarols, we have prepared novel dicoumarols (**1–10**) in high yield by reacting aldehydes with 4-hydroxycoumarin, in molar ratios of 1:2, in aqueous ethanol using piperidine as catalyst. Dicoumarols **1–10** were screened for their urease inhibitory activity. Urease (EC 3.5.1.5) is the enzyme that decomposes urea to ammonia and carbon dioxide. It is directly involved in the formation of

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renal stones, causing various urological disorders.[21,22] The ulcerogenic bacteria *Helicobacter pylori (HP)*, survives at low pH in the stomach, and ammonia generated by the urease encoded by this bacterium alters the local pH of the stomach environment, helping the pathogen to survive. This lowering in pH is suitable for survival of *Helicobacter pylori* but causes gastric and peptic ulcers to the host, which in some cases may degenerate to cancerous diseases.[22] Additionally, in agriculture, the large amount of ammonia evolution under the influence of different bacterial ureases causes environmental and economic problems due to the toxicity of ammonia and the diminution of nutrient supply for cultivated plants.[21,22] Since urease is not only damaging for humans, but also for animals and in agriculture, various strategies based on urease inhibition were considered as treatment approaches for infections caused by urease-producing bacteria.[21,22] Thus, the synthetic dicoumarols (**1–10**) reported here were tested for their urease inhibitory activity. To the best of our knowledge this is the first report of urease inhibitors belonging to this class of compounds.

MATERIALS AND METHODS

General

Melting points were determined on a Büchi 434 melting point apparatus and are uncorrected. NMR was performed on a Bruker AM 300 and 500 MHz. Ultraviolet spectra (UV) were recorded on a Perkin-Elmer Lambda-5 UV/VIS spectrometer in DMSO. Infrared Spectra (IR) was recorded on a JASCO IR-A-302 Spectrometer. Electron Impact Mass Spectra (EIMS) were recorded on a FINNIGAAN MAT-311A (Germany). CHN analysis was performed on a Carlo Erba Strumentazione-Mod-1106 (Italy). Thin layer chromatography (TLC) was performed on precoated TLC silica gel glass plates (Kieselgel 60, 254, E. Merck, Germany). Chromatograms were visualized by UV at 254 and 365 nm followed by iodine vapors.

Chemistry

General Procedure for the Synthesis of Compound (1–10)

To a mixture of aldehyde (1 mmol) and 4-hydroxycoumarin (2 mmol) dissolved in 10 ml of EtOH was added a few drops of piperidine and the mixture was stirred for 4 h at room temperature. After completion of the reaction (TLC monitored), water was added till precipitation. The precipitates were filtered and washed with ice-cooled water followed by ethanol and dried under vacuum.

3,3′-(3,4-Methoxybenzylidene)-Bis-(4-Hydroxycoumarin) (**1**)

Yield: 97%; M.p: 258°C; $R_f = 0.52$ (ethyl acetate); [Found: C, 68.69; H, 4.30. $C_{27}H_{20}O_8$ requires: C, 68.64%, H 4.27%]; IR (KBr): ν_{max} 3737 (OH), 1664 (C = O), 1612 (C = C), 1514 (C—O), cm^{-1}; UV (DMSO): λ_{max} 308.2 ($\varepsilon = 3.13$) nm; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.03 (*d*, 2H, $J = 8.1$ Hz, H-5/5′), 7.63 (*td*, 2H, $J = 8.1$, $J = 2.1$ Hz, H-7/7′), 7.40 (*d*, 2H, $J = 8.1$ Hz, H-8/8′), 7.35 (*td*, 2H, $J = 8.1$, $J = 2.5$ Hz, H-6/6′), 6.78 (*s*, 1H, H-2′), 6.68 (*d*, 1H, $J = 8.4$ Hz, H-6″), 6.66 (*d*, 1H, $J = 8.4$ Hz, H-5″), 6.00 (*s*, 1H, H-11), 3.81 (*s*, 3H, —OCH_3), 3.77 (*s*, 3H, —OCH_3); *m/z*: 472, 351, 310, 279, 252, 224,162,120, 92, 63.

3,3′-(3,4,5-Trimethoxybenzylidene)-Bis-(4-Hydroxycoumarin) (**2**)

Yield: 90%; M.p: 226°C; $R_f = 0.49$ (ethyl acetate); [Found: C, 66.91; H, 4.45. $C_{28}H_{22}O_9$ requires: C, 66.93%; H 4.41%]; IR (KBr): ν_{max} 3648 (OH), 2934, 1661 (C = O), 1617 (C = C), 1563 (C—O) cm^{-1}; UV (DMSO): λ_{max} 249.6 ($\varepsilon = 3.02$) nm; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.98 (*d*, 2H, $J = 8.0$ Hz, H-5/5′), 7.63 (*td*, 2H, $J = 8.0$, $J = 2.1$ Hz, H-7/7′), 7.39 (*d*, 2H, $J = 8.0$ Hz, H-8/8′), 7.30 (*td*, 2H, $J = 8.0$, $J = 2.1$ Hz, H-6/6′), 6.90 (*d*, 2H, $J = 1.4$ Hz, H-2″/6″), 6.07 (*s*, 1H, H-11), 3.83 (*s*, 3H, —OCH_3), 3.78 (s, 6H, —OCH_3); *m/z*: 502, 340, 309, 282, 267, 239, 174, 162, 120, 92, 63.

3,3′-(4-Ethoxybenzylidene)-Bis-(4-Hydroxycoumarin) (**3**)

Yield: 96%; M.p: 223°C: $R_f = 0.63$ (ethyl acetate); [Found: C, 71.10%, H, 4.39. $C_{27}H_{20}O_7$ requires: C, 71.05%; H 4.42%]; IR (KBr): ν_{max} 3047 (OH), 1677 (C = O), 1648 (C—O), 1532 (C = C) cm^{-1}; UV (DMSO): λ_{max} 302.6 ($\varepsilon = 3.15$) nm; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.78 (*d*, 2H, $J = 7.9$ Hz, H-5/5′), 7.62 (*td*, 2H, $J = 7.9$, $J = 1.6$ Hz, H-7/7′), 7.33 (*d*, 2H, $J = 7.9$ Hz, H-8/8′), 7.30 (*td*, 2H, $J = 7.9$, $J = 2.5$ Hz, H-6/6′), 7.22 (*d*, 2H, $J = 8.5$ Hz, H-2″/6″), 6.59 (*d*, 2H, $J = 8.4$ Hz, H-3″/5″), 6.15 (*s*, 1H, H-11), 3.8 (*q*, 2H, $J = 13.5$, $J = 6.6$ Hz, —O—CH_2—CH_3), 1.2 (*t*, 3H, $J = 6.6$ Hz, —O—CH_2—CH_3); *m/z*: 456, 294, 265, 250, 237, 162, 120, 92, 63.

3,3′-(4-Hydroxy, 3-Ethoxybenzylidene)-Bis-(4-Hydroxycoumarin) (**4**)

Yield: 98%; M.p.: 199°C; $R_f = 0.58$ (ethyl acetate); [Found: C, 68.67; H 4.28. $C_{27}H_{20}O_8$ requires: C 68.64%; H, 4.27%]; IR (KBr): ν_{max} 3625 (OH), 1670 (C = O), 1610 (C—O), 1528 (C = C), cm^{-1}; UV (DMSO): λ_{max} 303.1 ($\varepsilon = 3.07$) nm; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.80 (*d*, 2H, $J = 8.1$ Hz, H-5/5′), 7.57 (*td*, 2H, $J = 8.1$, $J = 2.4$ Hz, H-7/7′), 7.33 (*d*, 2H, $J = 8.1$ Hz, H-8/8′), 7.22 (*td*, 2H, $J = 8.1$, $J = 1.7$ Hz, H-6/6′), 7.05 (*d*, 2H, $J = 2.3$ Hz, H-2″), 6.98 (*d*, 1H, $J = 8.3$ Hz, H-6′), 6.55 (*d*, 1H, $J = 8.3$ Hz, H-5′), 5.96 (*s*, 1H, H-11), 3.77

(q, 1H, $J = 13.4$, $J = 6.4$ Hz, O—CH_2—CH_3), 1.16 (t, 3H, $J = 6.4$ Hz, O—CH_2—CH_3); m/z: 472, 351, 310, 298, 281, 265, 225, 162, 120, 92, 63.

3,3′-(4-Isopropylbenzylidene)-Bis-(4-Hydroxycoumarin) (5)

Yield: 94%; M.p: 239°C; $R_f = 0.67$ (ethyl acetate); [Found: C, 74.06; H, 4.86. $C_{28}H_{22}O_6$ requires: C, 74.00%, H 4.88%]; IR (KBr): ν_{max} 3057 (OH), 1670 (C = O), 1610 (C—O), 1528 (C = C) cm^{-1}; UV (DMSO): λ_{max} 306.2 ($\varepsilon = 3.16$) nm; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.80 (d, 2H, $J = 8.1$ Hz, H-5/5′), 7.57 (td, 2H, $J = 8.1$, $J = 1.5$ Hz, H-7/7′), 7.33 (d, 2H, $J = 8.1$ Hz, H-8/8′), 7.22 (td, 2H, $J = 8.1$, $J = 1.5$ Hz, H-6/6′), 7.05 (d, 2H, $J = 8.4$ Hz, H-2″/6″), 6.98 (d, 1H, $J = 8.4$ Hz, H-3″/5″), 6.10 (s, 1H, H-11), 2.77 (m, 1H, —$CH(CH_3)_2$), 1.16 (d, $J = 5.8$ Hz, 6H, —$(CH_3)_2$); m/z: 454, 335, 291,249, 162,120, 92, 63.

3,3′-(4-Dimethylaminobenzylidene)-Bis-(4-Hydroxycoumarin) (6)

Yield: 95%; M.p: 210°C; $R_f = 0.51$ (ethyl acetate); [Found: C, 71.16; H, 4.60; N, 3.13. $C_{27}H_{21}NO_6$ requires: C, 71.20; H, 4.65; N, 3.08%]; IR (KBr): ν_{max} 3030 (OH), 1665 (C = O), 1535 (C—O), 1043 (C—O) cm^{-1}; UV (DMSO): λ_{max} 307.0 ($\varepsilon = 3.17$) nm; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.83 (td, 2H, $J = 8.4$, $J = 2.0$ Hz, H-5/5′), 7.66 (2H, d, $J = 8.2$ Hz, H-2″/6″), 7.54 (td, 2H, $J = 8.4$, $J = 1.6$ Hz, H-7/7′), 7.29 (d, 2H, $J = 8.4$ Hz, H-8/8′), 7.25 (td, 2H, $J = 8.4$, $J = 2.1$ Hz, H-6/6′), 7.17 (d, 2H, $J = 8.2$ Hz, H-3″/5″), 5.98 (s, 1H, H-11), 2.49 (s, 6H, —$N(CH_3)_2$); m/z: 455, 336, 293,249, 215, 162,120, 92, 63.

3,3′-(4-Pyridyl-Methylene)-Bis-(4-Hydroxycoumarin) (7)

Yield: 97%; M.p: 218°C; $R_f = 0.69$ (ethyl acetate); [Found: C, 69.76; H, 3.60; N, 3.43. $C_{24}H_{15}NO_6$ requires: C, 69.73; H, 3.66; N, 3.39%]; IR (KBr): ν_{max} 3035 (OH), 1650 (C = O), 1620 (C—O), 1535 (C = C) cm^{-1}; UV (DMSO):λ_{max} 307.1 ($\varepsilon = 3.19$) nm; H^1-NMR (300 MHz, DMSO-d_6): δ 8.35 (d, 1H, $J = 8.6$ Hz, H-3″/5″), 7.92 (d, 2H, $J = 8.1$ Hz, H-5/5′), 7.67 (d, 1H, $J = 8.6$ Hz, H-2″/6″), 7.59 (td, 2H, $J = 8.1$, $J = 2.0$ Hz, H-7/7′), 7.35 (2H, d, $J = 8.1$ Hz, H-8/8′), 7.30 (td, 2H, $J = 8.1$, $J = 2.3$ Hz, H-6/6′), 5.98 (1H, s, H-11); m/z: 413, 317, 250, 222, 191, 162, 120, 92, 63.

3,3′-(3-Aminobenzylidene)-Bis-(4-Hydroxycoumarin) (8)

Yield: 97%; M.p: 214°C; $R_f = 0.57$ (ethyl acetate); [Found: C, 70.30; H, 3.96; N, 3.30. $C_{25}H_{17}NO_6$ requires: C, 70.25; H, 4.01; N, 3.28%]; IR (KBr); ν_{max} 3041 (OH), 1668 (C = O), 1648 (C—O), 1567 (C = C) cm^{-1}; UV (DMSO): λ_{max} 306.8 ($\varepsilon = 2.95$) nm; H^1-NMR (300 MHz, DMSO-d_6): δ 7.81 (d, 2H, $J = 7.9$ Hz, H-5/5′), 7.53 (td, 2H, $J = 7.9$, $J = 1.9$ Hz, H-7/7′), 7.35 (d, 1H, $J = 8.2$ Hz, H-6″), 7.30 (d, 2H, $J = 7.9$ Hz, H-8/8′), 7.22 (td, 2H, $J = 7.9$, $J = 1.8$ Hz, H-6/6′), 7.13 (s, 1H, H-2″), 7.02 (1H, td, J = 8.2, $J = 1.4$ Hz, H-5″), 6.90 (d, 1H, $J = 8.2$ Hz, H-4″), 6.08 (1H, s, H-11); m/z: 427, 267, 249, 187, 162, 120, 92, 63.

3,3′-(3-Nitrobenzylidene)-Bis-(4-Hydroxycoumarin) (9)

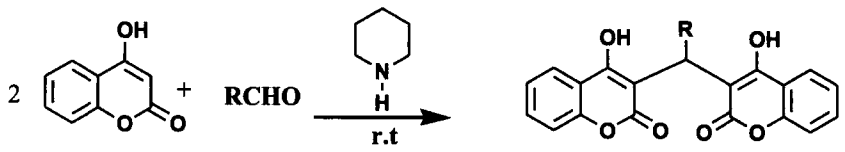
Yield: 94%; M.p: 214°C; $R_f = 0.56$ (ethyl acetate); [Found: C, 65.70; H, 3.35; N, 3.09. $C_{25}H_{15}NO_8$ requires: C, 65.65; H, 3.31; N, 3.06%]; IR (KBr): ν_{max} 3380 (OH), 2978 (N = O), 1678 (C = O), 1648 (C—O), 1540 (C = C) cm^{-1}; UV (DMSO): λ_{max} 306.8 ($\varepsilon = 3.16$) nm; H^1-NMR (300 MHz, DMSO-d_6): δ 7.81 (d, 2H, $J = 7.9$ Hz, H-5/5′), 7.53 (td, 2H, $J = 7.9$, $J = 2.0$ Hz, H-7/7′), 7.40 (d, 1H, $J = 8.4$ Hz, H-6″), 7.35 (d, 2H, $J = 7.9$ Hz, H-8/8′), 7.31 (s, 1H, H-2″), 7.22 (td, 2H, $J = 7.9$, $J = 1.6$ Hz, H-6/6′), 6.90 (1H, td, $J = 8.4$, $J = 1.7$ Hz, H-5″), 6.31 (d, 1H, $J = 8.4$ Hz, H-4″), 5.98 (s, 1H, H-11); m/z: 457,333, 295, 278, 248 162, 120, 93, 63.

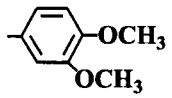
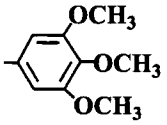
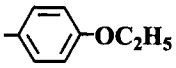
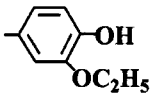
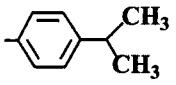
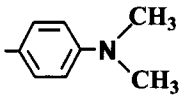
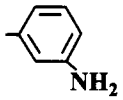
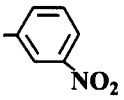
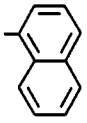
3,3′-(1-Naphthylidene)-Bis-(4-Hydroxycoumarin) (10)

Yield: 96%; M.p: 238°C; $R_f = 0.57$ (ethyl acetate); [Found: C, 75.40; H, 3.86. $C_{29}H_{18}O_6$ requires: C, 75.32; H, 3.92%]; IR (KBr): ν_{max} 3595 (OH), 1640 (C = O), 1631 (C—O), 1558 (C = C) cm^{-1}; UV (DMSO): λ_{max} 307.4 ($\varepsilon = 3.17$) nm; H^1-NMR (400 MHz, DMSO-d_6); δ 8.25 (d, 1H, $J = 8.5$ Hz, H-4″), 7.91 (d, 2H, $J = 8.5$ Hz, H-2″/8″), 7.86 (d, 2H, $J = 7.8$ Hz, H-5/5′), 7.68 (td, 2H, $J = 7.8$, $J = 2.3$ Hz, H-7/7′), 7.43 (d, 2H, $J = 7.8$ Hz, H-8/8′), 7.30–7.32 (m, 3H, H-3′/5′-7′), 7.26 (td, 2H, $J = 7.8$, $J = 1.6$ Hz, H-6/6′), 6.07 (1H, s, H-11); m/z: 462, 301, 215, 162, 162, 120, 93, 63.

Urease Assay and Inhibition

Reaction mixtures comprising 25 μL of enzyme (Jack bean urease, from Sigma-Aldrich, specific activity of 15 EU/mg) solution and 55 μL of buffer (0.01 M $K_2HPO_4 \cdot 3H_2O$, 1 mM EDTA and 0.01 M LiCl; pH 8.2) containing 100 mM urea were incubated with 5 μL of test compounds (0.01 μM–1 mM) dissolved in DMSO at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn.[23] Briefly, 45 μL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 μL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well (the final reaction volume was 200 μL) The increasing absorbance at 630 nm was measured after 50 min using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate. The results (change in absorbance per min) were processed by using SoftMax Pro software (Molecular Device, USA). Percentage inhibitions were calculated by using the formula



Compounds	R	Yield (%)	IC_{50} (microM)*
1		91	78.3
2		95	81.7
3		95	91.3
4		91	79.8
5		93	82.0
6		90	80.1
7		95	85.3
8		90	74.3
9		96	75.1
10		92	84.1

*Thiourea as standard shows an IC_{50} of 21μM under the same conditions.

SCHEME 1 Structures and the urease IC_{50} inhibition values for the dicoumarol derivatives.

$100-(OD_{testwell}/OD_{control} \times 100)$. Thiourea was used as the standard inhibitor of urease.[23]

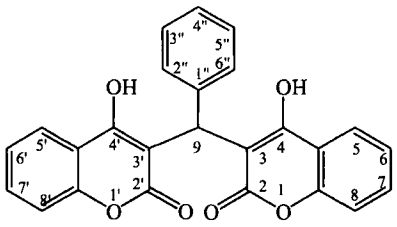
RESULTS AND DISCUSSION

Chemistry

The dicoumarols **1–10** were synthesized by condensing different aromatic aldehydes with 4-hydroxycoumarin at room temperature in the presence of catalytic amount of piperidine, working at 1:2 molar ratios. The yields in dicoumarols were high (Scheme 1).

The structures of the synthesized compounds described here were determined by using different spectroscopic techniques and purity was confirmed by CHN analysis. A general structure of the new dicoumarols is shown

below, with the numbering used in this article.



Urease Inhibition

During our preliminary studies on dicoumarols (unpublished results), it was found that some had inhibitory activity against urease and this initiated the synthesis of diversely substituted compounds of type **1–10** belonging to this class and testing them against urease, using thiourea as standard inhibitor, (IC_{50} value of 21 μM.[23])

All the synthesized dicoumarols (**1–10**) possessing different substituted aromatic residues at C-9 showed varying degrees of urease inhibitory activity. Compound **8** (IC_{50} = 74.31 μM) was the most potent urease inhibitor in this series of compounds. The second most active compounds was **9** (IC_{50} = 75.19 μM). Compounds **1**, **2** and **4** also containing *meta* substituted phenyl rings at C-9, demonstrated inhibitory activity in the range IC_{50} = 78.34–81.73 μM. The small decline in activity of compound **2** compared to compound **1** may be due to steric impairment due to substituents on adjacent carbons, which does not allow compound **2** to bind effectively within the enzyme active site. Compounds **3**, **5–7** and **10** also exhibited urease inhibitory effects IC_{50} = 80.12–91.35 μM. This entire class of dicoumarols shows urease inhibitory activity, and the differences between the diverse compounds **1–10** are rather small.

In summarizing the above findings, the best substitution pattern in the phenyl ring at C-9 includes compact substituents. The *meta*-substituted aromatic ring was the predominant factor in inducing urease inhibitory activity, whereas the corresponding *ortho*- and *para*- isomers were less effective. On the basis of these results, it may be considered that dicoumarols constitute interesting lead compounds for finding more effective urease inhibitors.

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