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MODIFIED COUMARINS. 27. SYNTHESIS AND ANTIOXIDANT ACTIVITY OF 3-SUBSTITUTED 5,7-DIHYDROXY-4-METHYLCOUMARINS

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Amides of 5,7-dihydroxy-4-methylcoumaryl-3-ylacetic acid were synthesized by the activated ester method using N-*hydroxysuccinimide and diisopropylcarbodiimide or by reaction with* N,N-*carbonylimidazole. The reactivity of 3-substituted 5,7-dihydroxy-4-methylcoumarins toward 2,2-diphenyl-1-picrylhydrazyl and superoxide radical was analyzed. The effect of the synthesized compounds on xanthineoxidase activity was studied.*

Key words: coumarins, antioxidants, xanthineoxidase, active oxygen species.

Natural coumarins and their synthetic structural analogs possess a broad spectrum of biological activity including anti-inflammatory, antibacterial, and analgesic action [1, 2]. The design of new derivatives of benzopyran-2-one is often based on research of the functions of these compounds in model systems. It has been found that simple natural coumarins containing two ortho hydroxyls (6,7-dihydroxycoumarins, 7,8-dihydroxycoumarins) are effective traps for the superoxide radical and alkylperoxyl radicals and are inhibitors of peroxide oxidation of lipids. However, these compounds in the presence of iron ions can also be prooxidants that are capable under certain conditions of stimulating undesired redox transformations involving molecular oxygen. Therefore, coumarin derivatives containing meta dihydroxyls are definitely interesting for constructing potential synthetic antioxidants. Thus, 5,7-dihydroxy-4-methylcoumarin is not a pro-oxidant, inhibits peroxide oxidation of lipids, and reacts with active oxygen species and hypochlorite [3]. Besides, it inhibits transformations catalyzed by cyclooxygenase and does not affect the activity of 5-lipoxygenase [2, 4, 5].

Because of the importance of the 5,7-dihydroxy-4-methylcoumarin moiety in the structure of a potential antioxidant, we attempted to analyze certain properties of 5,7-dihydroxy-4-methylcoumarins containing 3-substituents. A series of structurally similar amides was synthesized using 5,7-dihydroxy-4-methylcoumarin-3-ylacetic acid as starting material. Their reactivity toward 2,2-diphenyl-1-picrylhydrazyl and superoxide radical was studied. The effect of the synthesized compounds on xanthineoxidase activity was also found.

5,7-Dihydroxy-4-methylcoumarin-3-ylacetic acid (**2**) that was required for subsequent transformations was prepared by saponification of the ester in 5,7-dihydroxy-4-methylcoumarin (**1**) by NaOH in aqueous propan-2-ol with subsequent acidolysis of the reaction mixture. Coumarin **1** was synthesized by condensation of phloroglucinol dihydrate and dimethylacetylsuccinate in the presence of dry HCl.

Amides of **2** were prepared by two methods. The first used *N*-acylation based on the activated ester method that is used commonly in peptide synthesis [6]. The carboxylic acid was activated using a highly reactive *N*-hydroxysuccinimide ester [7]. The activated ester was prepared by reacting **2** and *N*-hydroxysuccinimide (SuOH) in absolute dioxane using diisopropylcarbodiimide (DIC) as the condensing agent. Condensation of the resulting activated ester with amines in dioxane at room temperature formed amides **3**-**11** in high yields.

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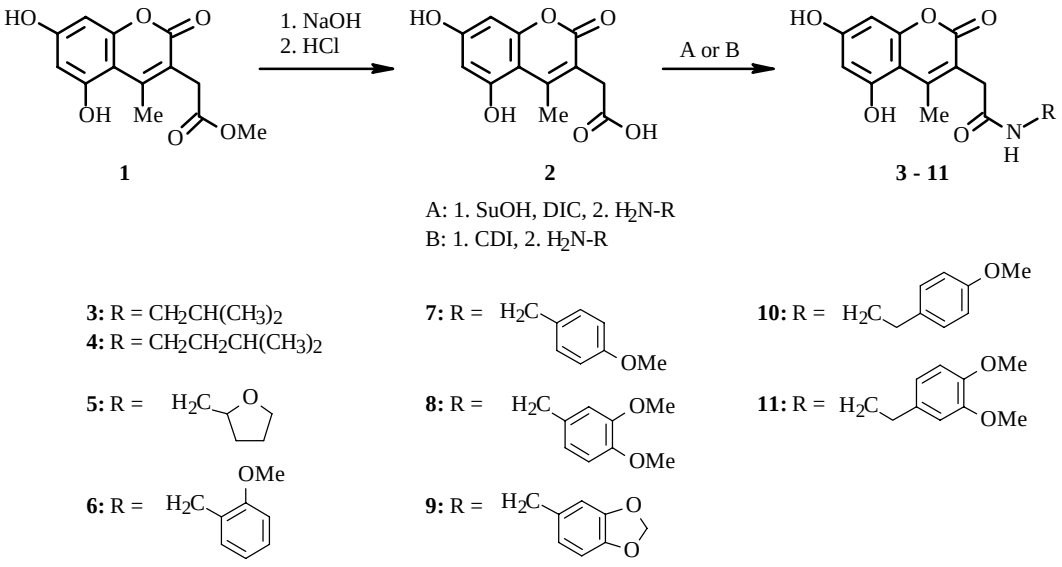
TABLE 1. Antioxidant Properties of 3-Substituted 5,7-Dihydroxy-4-methylcoumarins **2-11**

Compound	Effect on superoxide-dependent reduction of ferricytochrome C*	Relative reactivity toward DPPH**
2	0.84±0.11	1.0±0.2
3	0.71±0.07	2.3±0.2
4	0.78±0.14	2.2±0.1
5	0.73±0.04	1.7±0.2
6	0.74±0.03	2.3±0.7
7	0.62±0.14	2.0±0.6
8	0.43±0.06	1.6±0.1
9	0.60±0.05	2.2±0.4
10	0.63±0.07	2.4±0.9
11	0.53±0.07	1.8±0.5

*Ratio of initial rates of reduction of ferricytochrome C on reaction with superoxide with and without antioxidant.
**Ratio of initial rates of conversion of DPPH with the studied compound and 5,7-dihydroxy-4-methylcoumarin.



The second method was based on activation of the carboxylic acid by *N*,*N*-carbonyldiimidazole (CDI) [8, 9]. Reaction of **2** and CDI in absolute DMF formed *N*-acylimidazole, which reacted with the amines to produce in high yields the corresponding amides **3-11**.

The antioxidant properties of **3-11** were compared with those of **2** and 5,7-dihydroxy-4-methylcoumarin.

The ability of **3-11** to capture free radicals was evaluated by recording the rate of reaction of these compounds with 2,2-diphenyl-1-picrylhydrazyl. The results (Table 1) indicated that the reactivity of acid **2** was unchanged compared with 5,7-dihydroxy-4-methylcoumarin. However, it about doubled on going to amides **3-11**. The nature of the amide fragment had little effect on the manifestation of their antiradical activity.

The reactivity of the coumarins toward superoxide radical generated under anaerobic conditions in a system with xanthineoxidase and xanthine was evaluated by competitive reduction of ferricytochrome C. It was found in prior experiments that the rate of reduction of ferricytochrome C in the presence of 5,7-dihydroxy-4-methylcoumarin ($1 \cdot 10^{-4}$ M) was 50 ± 6% of the rate without antioxidant. Table 1 shows that the effect of **2** and **3-6** is less significant. However, **7-11** inhibit the reaction more and **8** decreases the reduction rate of ferricytochrome C about the same as 5,7-dihydroxy-4-methylcoumarin.

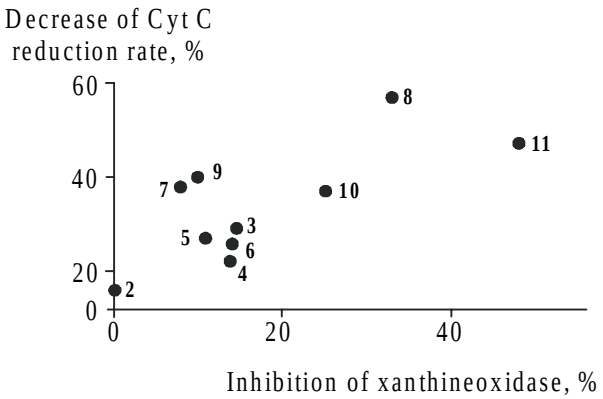


Fig. 1. Activity of **2-11** in xanthine-xanthineoxidase superoxide-generating system (% inhibition of initial rate of superoxide-dependent reduction of ferricytochrome C) as a function of inhibiting properties of these compounds toward xanthineoxidase (% inhibition of initial rate of enzymatic reaction).

The studied coumarins also inhibited xanthineoxidase, which is used in model systems to generate superoxide radical. An analysis of the effect of 3-substituted 5,7-dihydroxy-4-methylcoumarins on xanthineoxidase activity showed that the reduction in the rate of transformation of xanthine into uric acid in the presence of **2-11** depended on the structure of the inhibitor. Acid **2** practically did not inhibit the enzyme whereas **3-11** at concentrations of 100 μM decreased the rate of the enzymatic reaction by 10-50%.

It can be assumed that the observed effects of **3-11** during reduction of ferricytochrome C (Table 1) are caused partially by inhibition of the enzymatic reaction and, therefore, reduction of the rate of formation of oxygen anion-radical. Figure 1 shows a possible dependence of the inhibition by the studied compounds on xanthineoxidase and their effect on the rate of superoxide reduction of ferricytochrome C in the presence of this enzyme. Obviously the properties of **3-11** are due primarily to the presence of the 5,7-dihydroxy-4-methylcoumarin moiety, which is responsible for direct capture of superoxide radical and for binding of the inhibitor at the active center of xanthineoxidase. The nature of the amide fragment in **3-11** can substantially change the affinity of the inhibitor for the enzyme during formation of the complex with xanthineoxidase. Thus, **3-6** relatively weakly inhibit the formation of oxygen anion-radical and affect superoxide-dependent reduction of ferricytochrome C, similar to **2**. Introducing a 3,4-dimethoxybenzyl or 3,4-dimethoxyphenylethyl substituent into the antioxidant structure (**8** and **11**) markedly increases the inhibitory activity toward xanthineoxidase and effectively decreases the observed rate of superoxide-dependent reduction of ferricytochrome C.

Thus, the amides of 5,7-dihydroxy-4-methylcoumarin-3-ylacetic acid synthesized by us could inhibit xanthineoxidase and exhibit antiradical activity by reacting with DPPH and superoxide radical.

EXPERIMENTAL

The course of reactions and the purity of products were monitored by TLC on Merck 60 F254 plates with elution by $CHCl_3$:CH_3OH (9:1 and 95:5). Melting points were determined on a Kofler block. PMR spectra were recorded on Varian VXR-300 (300 MHz) and Varian Mercury 400 (400 MHz) spectrometers relative to TMS (internal standard). Elemental analyses of all compounds agree with those calculated. Spectrophotometric measurements were made on a Specord M-40 instrument.

5,7-Dihydroxy-4-methylcoumarin was prepared by Pechmann condensation of phloroglucinol and ethylacetoacetate in the presence of conc. H_2SO_4 [10]. We used buttermilk xanthineoxidase (Sigma), xanthine (Sigma), 2,2-diphenyl-1-picrylhydrazyl (Sigma), and cytochrome C from horse heart (Serva).

Methyl-(5,7-dihydroxy-4-methyl-2-oxochromen-3-yl)acetate (1). A stream of dry HCl was passed through a cold (0°C) solution of phloroglucinol dihydrate (16.21 g, 0.1 mol) and dimethylacetylsuccinate (18.82 g, 0.1 mol) in methanol

(50 mL) for 3 h with vigorous stirring and cooling. The mixture was left overnight at room temperature and poured into icewater (500 mL). The resulting precipitate was filtered off and recrystallized from methanol. Yield 86%, mp 259-260°C, $C_{13}H_{12}O_6$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.49 (3H, s, CH_3-4), 3.60 (2H, s, CH_2-3), 3.61 (3H, s, $COOC\underline{H}_3$), 6.18 (1H, d, J = 2.0, H-6), 6.29 (1H, d, J = 2.0, H-8), 10.29 and 10.57 (2H, two s, OH-5 and OH-7).

(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)acetic Acid (2). A solution of **1** (21.14 g, 80 mmol) in propan-2-ol (50 mL) was treated with a solution of NaOH (12.80 g, 320 mmol) in water (150 mL). The mixture was heated and stirred vigorously for 1 h (TLC) and acidified to pH 4. The resulting precipitate was filtered off and recrystallized from aqueous methanol. Yield 78%, mp 271-272°C, lit. [11] mp 264°C, [12] 285°C, $C_{12}H_{10}O_6$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.47 (3H, s, CH_3-4), 3.60 (2H, s, CH_2-3), 6.15 (1H, d, J = 2.0, H-6), 6.27 (1H, d, J = 2.0, H-8), 10.29 and 10.57 (2H, two s, OH-5 and OH-7), 11.50 (1H, br.s, COOH).

General Method of Synthesizing Amides 3-11. Method A. A solution of **2** (0.75 g, 3 mmol) and SuOH (0.38 g, 3.3 mmol) in absolute dioxane (10 mL) was stirred vigorously, treated with DIC (0.52 mL, 3.3 mmol), and stirred for 2 h (course of reaction monitored by TLC). The resulting activated ester was treated with the appropriate amine (3.3 mmol). The mixture was stirred vigorously for 4-6 h (reaction monitored by TLC). After the reaction was complete, the mixture was diluted with water (100 mL) and acidified to pH 5-6. The resulting precipitate was filtered off and crystallized from propan-2-ol.

Method B. A solution of **2** (0.75 g, 3 mmol) in absolute DMF (5 mL) was treated with CDI (0.54 g, 3.3 mmol), stirred vigorously at room temperature for 2 h, treated with the appropriate amine (3.3 mmol), and stirred vigorously at room temperature for 4-6 h. After the reaction was complete, the mixture was diluted with water (100 mL) and acidified to pH 5-6. The resulting precipitate was filtered off and crystallized from propan-2-ol.

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-isobutylacetamide (3). Yield 68%, mp 142-143°C, $C_{16}H_{19}NO_5$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 0.80 [6H, d, J = 6.4, $CH_2CH(C\underline{H}_3)_2$], 1.66 [1H, m, $CH_2C\underline{H}(CH_3)_2$], 2.44 (3H, s, CH_3-4), 2.84 [2H, m, $C\underline{H}_2CH(CH_3)_2$], 3.35 (2H, s, CH_2-3), 6.14 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 7.81 (1H, t, J = 5.6, CONH), 10.21 and 10.47 (2H, two s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-isopentylacetamide (4). Yield 72%, mp 156-157°C, $C_{17}H_{21}NO_5$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 0.83 [6H, d, J = 6.0, $CH_2CH_2CH(C\underline{H}_3)_2$], 1.26 [2H, m, $CH_2C\underline{H}_2CH(CH_3)_2$], 1.53 [1H, m, $CH_2CH_2C\underline{H}(CH_3)_2$], 2.43 (3H, s, CH_3-4), 3.03 [2H, m, $C\underline{H}_2CH_2CH(CH_3)_2$], 3.36 (2H, s, CH_2-3), 6.14 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 7.76 (1H, t, J = 5.2, CONH), 10.30 (2H, br.s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-(tetrahydrofuran-2-ylmethyl)acetamide (5). Yield 79%, mp 176-177°C, $C_{17}H_{19}NO_6$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 1.44-1.82 (4H, m, CH_2-3′, CH_2-4′), 2.43 (3H, s, CH_3-4), 3.09 (2H, m, $NHC\underline{H}_2$), 3.39 (2H, s, CH_2-3), 3.55-3.81 (3H, m, H-2′, CH_2-5′), 6.14 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 7.90 (1H, t, J = 5.6, CONH), 10.22 and 10.47 (2H, two s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-(2-methoxybenzyl)acetamide (6). Yield 82%, mp 192-193°C, $C_{20}H_{19}NO_6$.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.50 (3H, s, CH_3-4), 3.45 (2H, s, CH_2-3), 3.80 (3H, s, OCH_3), 4.20 (2H, d, J = 5.7, $NHC\underline{H}_2$), 6.12 (1H, d, J = 2.1, H-6), 6.23 (1H, d, J = 2.1, H-8), 6.82-6.94 (2H, m, H-3′, H-5′), 7.12-7.22 (2H, m, H-4′, H-6′), 8.02 (1H, t, J = 5.7, CONH), 10.05 and 10.30 (2H, two s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-(4-methoxybenzyl)acetamide (7). Yield 91%, mp 221-222°C, $C_{20}H_{19}NO_6$.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.50 (3H, s, CH_3-4), 3.42 (2H, s, CH_2-3), 3.73 (3H, s, OCH_3), 4.17 (2H, d, J = 5.7, $NHC\underline{H}_2$), 6.11 (1H, d, J = 2.1, H-6), 6.23 (1H, d, J = 2.1, H-8), 6.82 (2H, d, J = 8.7, H-3′, H-5′), 7.14 (2H, d, J = 8.7, H-2′, H-6′), 8.17 (1H, t, J = 5.7, CONH), 10.00 and 10.26 (2H, two s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-(3,4-dimethoxybenzyl)acetamide (8). Yield 86%, mp 216-217°C, $C_{21}H_{21}NO_7$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.47 (3H, s, CH_3-4), 3.45 (2H, s, CH_2-3), 3.71 (6H, s, two OCH_3), 4.17 (2H, d, J = 5.6, $NHC\underline{H}_2$), 6.15 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 6.75-6.86 (3H, m, H-2′, H-5′, H-6′), 8.27 (1H, t, J = 5.6, CONH), 10.21 and 10.47 (2H, two s, OH-5 and OH-7),

***N*-(1,3-Benzodioxol-5-ylmethyl)-2-(5,7-dihydroxy-4-methyl-2-oxochromen-3-yl)acetamide (9).** Yield 88%, mp 202-203°C, $C_{20}H_{17}NO_7$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.45 (3H, s, CH_3-4), 3.43 (2H, s, CH_2-3), 4.14 (2H, d, J = 6.0, NHC$\underline{H}_2$), 5.96 (2H, s, OCH_2O), 6.15 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 6.69-6.83 (3H, m, H-4′, H-6′, H-7′), 8.29 (1H, t, J = 5.6, CONH), 10.30 (2H, br.s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-[2-(4-methoxyphenyl)ethyl]acetamide (10). Yield 81%, mp 195-196°C, $C_{21}H_{21}NO_6$.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.40 (3H, s, CH_3-4), 2.60 (2H, t, J = 7.2, NHCH_2C$\underline{H}_2$), 3.20 (2H, m, NHC$\underline{H}_2$CH_2), 3.50 (2H, s, CH_2-3), 3.69 (3H, s, OCH_3), 6.15 (1H, d, J = 2.0, H-6), 6.26 (1H, d, J = 2.0, H-8), 6.81 (2H, d, J = 8.4, H-3′, H-5′), 7.08 (2H, d, J = 8.4, H-2′, H-6′), 7.82 (1H, t, J = 5.2, CONH), 10.21 and 10.47 (2H, two s, OH-5 and OH-7).

2-(5,7-Dihydroxy-4-methyl-2-oxochromen-3-yl)-*N*-[2-(3,4-dimethoxyphenyl)ethyl]acetamide (11). Yield 89%, mp 206-207°C, $C_{22}H_{23}NO_7$.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.44 (3H, s, CH_3-4), 2.63 (2H, t, J = 7.2, NHCH_2C$\underline{H}_2$), 3.25 (2H, m, NHC$\underline{H}_2$CH_2), 3.35 (2H, s, CH_2-3), 3.71 and 3.75 (6H, two s, two OCH_3), 6.11 (1H, d, J = 2.1, H-6), 6.23 (1H, d, J = 2.1, H-8), 6.62-6.79 (3H, m, H-2′, H-5′, H-6′), 7.69 (1H, t, J = 5.6, CONH), 10.01 and 10.27 (2H, two s, OH-5 and OH-7).

Superoxide-dependent Reduction of Ferricytochrome C. Xanthine (50 μM), ferricytochrome C (20 μM), 5,7-dihydroxy-4-methylcoumarin (**2-11**, 0.1 mM), and EDTA (0.1 mM) were dissolved in sodium-phosphate buffer (50 mM, pH 7.4). A working solution of **3-11** in DMSO was prepared and added slowly to the buffer. Derivative **2** was dissolved beforehand in the same amount of ethanol. The concentration (by volume) of organic solvent was 1%. The reaction was started by adding xanthineoxidase (0.004 units/mL in the mixture). The rate of reduction of ferricytochrome C at 25°C was monitored by measuring the optical density at 550 nm.

Effect of 2-11 on Xanthineoxidase Activity. The enzymatic reaction was investigated in sodium-phosphate buffer (50 mM, pH 7.4) at 25°C. The mixture contained xanthine (50 μM), xanthineoxidase (0.004 units/mL), coumarin (0.1 mM, **2-11**), EDTA (0.1 mM), and DMSO (1 vol%). The reaction rate was monitored by the change of optical density at 295 nm.

Reaction of 2-11 with 2,2-Diphenyl-1-picrylhydrazyl. The reaction was studied in ethanol containing coumarin (0.1 mM) and DPPH (50 μM). The mixture was stirred, thermostatted at 25°C, and monitored by the change of optical density at 517 nm.

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