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3-HYDROXYCOUMARINS: FIRST DIRECT PREPARATION FROM COUMARINS USING A Cu^{2+} – ASCORBIC ACID – O_2 SYSTEM, AND THEIR POTENT BIOACTIVITIES[1]

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SUMMARY: First direct 3-hydroxylation of a coumarin ring *via* a purely chemical system, previously only possible using cytochrome P-450, was successfully conducted by a Cu^{2+} – ascorbic acid – O_2 system; the two 3-hydroxycoumarins obtained were novel compounds, 3-hydroxyscopoletin and 3-hydroxyisoscopoletin. 5-Lipoxygenase and α-D-glucosidase inhibitory activities of coumarins greatly increased through 3-hydroxylation. 3-Hydroxyscopoletin and 3-hydroxyumbelliferone had a high inhibitory potency for 5-lipoxygenase and for α-D-glucosidase respectively; they serve as lead compounds for new drugs.

A number of drugs and xenobiotics are known to be deactivated or detoxicated through metabolic reaction which proceeds mainly *via* use of cytochrome P-450 (1,2). However, this process presents serious problems in medicinal chemistry in that some of these substances are converted to more bioactive or toxic metabolites (1); moreover the ultimate activated metabolites of some drugs are unclear as yet.

Many crude or synthesized drugs contain a coumarin ring structure. Previous reports have described that, by rat-liver microsomes, coumarin mainly undergoes hydroxylation at the 3-position which depends on cytochrome P-450 (3-5). Nevertheless, known 3-hydroxycoumarins in nature are quite rare (6,7) and their bioactivities have never been assayed yet. Thus, not only is the investigation itself of bioactivities of various 3-hydroxycoumarins of great value, but, through comparison with the activities of their precursors, is also useful in searching for the active

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Abbreviations used: TLC, thin layer chromatography; HPLC, high performance liquid chromatography; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance, mp, melting point; dec., decomposition; sp, subliming point; TMS, tetramethylsilane; br., broad peak; Ar, aromatic ring; sh, shoulder peak.

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metabolites of the drugs containing a coumarin ring. Hitherto, to our knowledge, 3-hydroxylation of a coumarin ring, effectively possible using the the P-450 system, has not been reported to proceed in purely chemical systems.[3]

We recently reported that, by using a Cu^{2+} – ascorbic acid – O_2 system, oxidative conversion of *o*-methoxyphenols was successfully conducted to selectively afford the corresponding catechols which suffered no further oxidation (9-12).

The Cu^{2+} – ascorbic acid – O_2 system is often utilized as a system of generating active oxygen species *in vitro* to degrade biomolecules (13-21) such as amino acids, proteins and DNA; as a synthetic tool, however, there has been no report of its use other than as described in our work (9-12).

In the present communication, we report that direct 3-hydroxylation of a coumarin ring was effected for the first time with a chemical oxidation system through application of this oxidation couple to coumarin compounds; further, the 5-lipoxygenase of rat basophilic leukemia cells and α-D-glucosidase inhibitory activities of the 3-hydroxycoumarins obtained were much enhanced in comparison with those of their precursors.

MATERIALS AND METHODS

Scopoletin, esculetin, and 4-methylesculetin were purchased from Aldrich Chemical Company, Inc.; umbelliferone and 4-methylumbelliferone from Tokyo Kasei Kogyo Co., Ltd.; 4-hydroxycoumarin and cupric perchlorate hexahydrate from Nacalai Tesque, Inc.; ascorbic acid from Wako Pure Chemical Industries Ltd. Isoscopoletin was provided by Professor Ushio Sankawa. Acetone was distilled over potassium permanganate. Other reagents were the best grades commercially available. TLC was purchased from Merck Co., Ltd. (Silicagel 60 F_{254}, No. 5554).

The coumarin oxidations were carried out as follows, except when otherwise noted. A yellow clear solution of a coumarin compound (0.1 mmol), cupric perchlorate hexahydrate (0.1 mmol), and ascorbic acid (1.0 mmol) in acetic acid (1.5 ml) – water (2 ml) was stirred vigorously for 4 h under pure oxygen atmosphere at room temperature. After the addition of methanol and an internal standard solution to the reaction mixture, the products were analyzed by HPLC and the yields were based on the substrate. All products and the unreacted substrate were isolated and characterized by several instrumental analyses.

HPLC was performed with a reversed phase column (Partisil-5 ODS-3, 4.3 × 150 mm) under the following conditions: for scopoletin, isoscopoletin, and umbelliferone as a substrate, eluent [acetonitrile : 0.1% H_3PO_4aq. = 1 : 4 v/v], flow rate [1.0 ml/min], detection UV [298 nm]; for 4-methylesculetin, eluent [acetonitrile : 0.1% H_3PO_4aq. = 1 : 3 v/v], flow rate [1.5 ml/min], detection UV [310 nm].

Isolations were carried out under the following procedures. The reaction mixture was extracted with ethyl acetate three times and the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and then evaporated under reduced pressure to obtain the pale yellow powder, which was subjected to silica gel (Merck Co., Ltd., Silicagel 60, No. 9385) column chromatography (eluent; dichloromethane : ethyl acetate = 9 : 1 – 2 : 1) to yield the products.

Elemental analyses were performed by the Microanalytical Laboratory at the University of Tokyo. Melting points were determined on a Yanagimoto micro melting point apparatus and are

[3]Coumarin can be hydroxylated at the 7-position to obtain umbelliferone **6** by a chemical system (8).

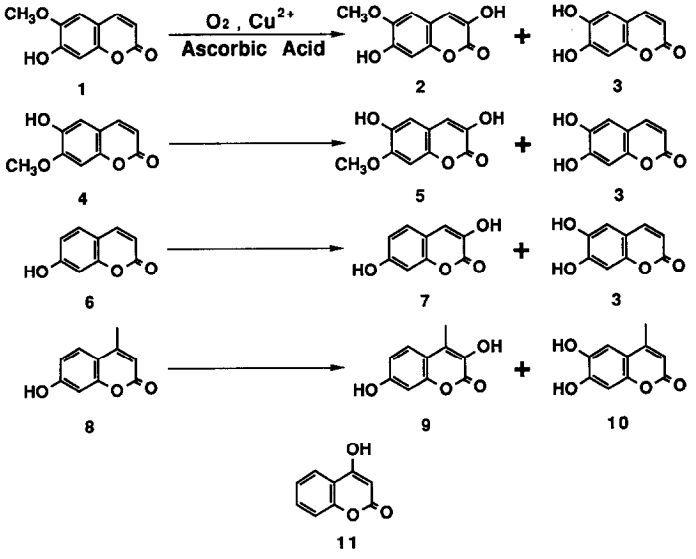
uncorrected. Mass spectra were measured on a JEOL DX-300 mass spectrometer operating in the direct injection-electron impact mode. IR spectra were recorded on a JASCO DS-701G spectrophotometer. NMR spectra were recorded on a JEOL GSX-400 (400 MHz) pulse Fourier-transform NMR spectrometer. Chemical shifts are expressed in units (ppm) downfield of internal tetramethylsilane.

5-Lipoxygenase used was the 10,000×g or 105,000×g supernatant from the centrifuged mixture after homogenization and sonication of rat basophilic leukemia cells. Assays to determine 5-lipoxygenase activity were performed at 37°C for 5 min in 200-μl incubation mixtures composed of phosphate buffer (100 mM, pH 7.4), reduced glutathione (1.0 mM), ATP (2.0 mM), $CaCl_2$ (2.0 mM), the enzyme, and various concentrations of test compounds. Reactions were initiated by addition of 1-^{14}C-arachidonic acid (30 μM, 35,000 cpm) and terminated by acidification with an aqueous solution of citric acid (0.2 μM, 20 μl) and extraction with ethyl acetate (300 μl). After evaporation of the extracts (200 μl), reaction products of each sample were separated by TLC (Whatman 60A LK6D) *via* use of the mobile phase (the mixed solvent composed of ether : petroleum ether : acetic acid = 50 : 50 : 1) and measured by liquid scintillation spectroscopy. Inhibition of 5-lipoxygenase activity was calculated as the ratio of the product formed in the presence and absence of inhibitor.

α-D-Glucosidase was purchased from Sigma Chemical Co., St. Louis, MO (α-glucosidase; yeast; maltase, α-D-glucoside glucohydrolase type I). The enzyme activity was determined spectrophotometrically using *p*-nitrophenyl α-D-glucopyranoside (Sigma Chemical Co., St. Louis, MO) as a specific substrate for this enzyme. A 100-μl mixed solution composed of phosphate buffer (100 mM, pH 6.8), the substrate (0.66 mM), and the enzyme (0.17 μg) was incubated at 37°C for 15 min with different concentrations of the test compound. The production of a yellow *p*-nitrophenolate anion was followed spectrophotometrically at 405 nm. Inhibition of α-D-glucosidase activity was calculated as the ratio of product formed in the presence and absence of inhibitor.

RESULTS AND DISCUSSIONS

As Scheme 1 shows, by the Cu^{2+}– ascorbic acid – O_2 system, scopoletin **1** and isoscopoletin **4** were regioselectively hydroxylated at the 3-position to give 3-hydroxyscopoletin **2**



Scheme 1

Table I. 3-Hydroxylation and Catechol Formation of Coumarins **1**, **4**, **6**, and **8** by a Cu^{2+} – Ascorbic Acid – O_2 System

Substrate	Products, yield			
	3-OH compd[a]		Catechol compd[b]	
Scopoletin **1**	**2**	2%	**3**	5%
Scopoletin **1**[c]	**2**	19	**3**	5
Isoscopoletin **4**	**5**	12	**3**	4
Umbelliferone **6**	**7**	16	**3**	2
4-Methylumbelliferone **8**	**9**	tr.[d]	**10**	13

Reaction conditions are described in METHODS.
a. 3-Hydroxylated compound. b. Catechol compound. c. This reaction was carried out under following conditions: after the addition of an aqueous solution of ascorbic acid (13.2 mmol) in water (10 ml) to a blue clear solution of scopoletin **1** (0.63 mmol) and cupric perchlorate hexahydrate (0.63 mmol) in acetone (10 ml), the yellow clear solution was stirred vigorously under oxygen atmosphere at room temperature; to the reaction mixture, more ascorbic acid (13.2 mmol) in water (10 ml) and the copper salt (0.63 mmol) was added twice at four-hour intervals from the reaction start. After the solution was stirred 15 hours more, the products were isolated as described in METHODS. d. Trace amount.

and 3-hydroxyisoscopoletin **5**, respectively, at the same time when both underwent catechol formation to afford the same product, esculetin **3** (Table I). This was the first example of 3-hydroxylation of a coumarin ring proceeding with a purely chemical oxidation system. The use of *m*-chloroperbenzoic acid either with or without *meso*-tetrakis(2,6-difluorophenyl)-porphyrinatoiron(III) chloride gave no oxidized products of 7-ethoxycoumarin although in both cases epoxidization of olefins is fully possible. Two novel 3-hydroxycoumarins **2** and **5** were obtained and their structures were characterized by NMR, IR, mass spectra, and elemental analysis.[4,5]

[4]3-Hydroxyscopoletin (3,7-dihydroxy-6-methoxycoumarin) **2** exists as colorless needles with mp 260°C (dec.) and sp 230°C; ^{1}H-NMR (400 MHz; CD_3COCD_3/TMS) δ 8.3 (br. s, 1H, exchangeable with D_2O; C(7)-OH), 7.35 (br. s, 1H, exchangeable with D_2O; C(3)-OH), 7.08 (s, 2H; C(4)-H and C(5)-H), 6.81 (s, 1H; C(8)-H), 3.89 (s, 3H; OCH_3); ^{13}C-NMR (100.5 MHz; Complete decoupling/CD_3COCD_3/The center peak of $\underline{C}D_3COCD_3$ (29.8 ppm) is used as a reference.) δ 160.2 (C=O), 148.4 (C(Ar)-O), 146.3 (C(Ar)-O), 145.7 (C(Ar)-O), 140.0 (C(3)-O), 116.2 (C(4) or C(5)), 113.0 (C(4a)), 108.7 (C(4) or C(5)), 103.4 (C(8)), 56.7 (CH_3O); IR (KBr) 3470 cm^{-1} and 3430sh and 3350 (OH), 3050 and 2920 and 2850 (CH), 2320, 1703sh and 1691 (C=O), 1648, 1625, 1591, 1510, 1478, 1446, 1425; Mass spectrum m/z = 208 (M^+), 193 (M^+–CH_3), 180, 165, 137; Anal. Calcd for $C_{10}H_8O_5$: C, 57.70; H, 3.87. Found: C, 57.41; H, 3.85.

[5]3-Hydroxyisoscopoletin (3,6-dihydroxy-7-methoxycoumarin) **5** exists as slightly yellow needles with mp 225°C (dec.) and sp 205°C; ^{1}H-NMR (400 MHz; CD_3COCD_3/TMS) δ 8.25 (br. s, 1H; C(6)-OH), 7.97 (br. s, 1H; C(3)-OH), 7.05 (s, 1H; C(5)-H), each signal of 6.95 (s, 1H) and 6.94 (s, 1H) was assigned to C(4)-H or C(8)-H, 3.93 (s, 3H; OCH_3); ^{13}C-NMR (100.5 MHz; Complete decoupling/CD_3COCD_3/The center peak of $\underline{C}D_3COCD_3$ (29.8 ppm) is used as a reference.) δ 160.2 (C=O), 149.1 (C(Ar)-O), 144.7 (C(Ar)-O), 140.5 (C(Ar)-O), 129.1 (C(3)-O), 115.8 (C(4) or C(5)), 114.3 (C(4a)), 111.4 (C(4) or C(5)), 100.4 (C(8)), 56.6 (CH_3O); IR (KBr) 3510 cm^{-1} and 3330 (OH), 3050 and 2920 and 2840 (CH), 2300, 1688sh and 1678 (C=O), 1643, 1624, 1580, 1504, 1451, 1409; Mass spectrum m/z = 208 (M^+), 193 (M^+–CH_3), 165, 149. Anal. Calcd for $C_{10}H_8O_5$: C, 57.70; H, 3.87. Found: C, 57.48; H, 3.87.

Two umbelliferones were also hydroxylated. Umbelliferone **6** underwent hydroxylation at two positions to give 3-hydroxyumbelliferone **7** and the 6-hydroxylated compound, esculetin **3**. The instrumental analytical data of 3-hydroxyumbelliferone **7** are in accord with those of the natural product (6). Nevertheless, 4-methylumbelliferone **8** was converted selectively into the 6-hydroxylated product, 4-methylesculetin **10** (Table I). The 6-hydroxylation of the umbelliferones is a rare example of the *ortho* selective phenol hydroxylation which can also be catalyzed by cytochrome P-450 (22-24); however, this has not yet been efficiently conducted by any chemical oxidation systems (25-27). This oxidation couple can serve as a synthetic tool to prepare the metabolites of coumarins directly.

In these coumarin oxidations, any other remarkable compounds were not detectable by HPLC besides the starting material recovered. These regioselective conversions are presumed to result from coordination of a phenolic hydroxy group or a carbonyl group to the copper ion and subsequent selective oxidation at the vicinal site by active oxygen species generated on the metal ion.

These reactions also proceeded with hydrogen peroxide instead of molecular oxygen to yield the same products. Thus, we presume that the ultimate oxidative species in this reaction is generated from dioxygen *via* formation of hydrogen peroxide.

The 5-lipoxygenase inhibitory activities of 3-hydroxyled or catechol derivatives and their precursors were assayed (Table II), and the activities of the 3-hydroxylcoumarins and catechols were much higher than those of their precursors. These new bioactive 3-hydroxycoumarins **2**, **5**, and **7** show promise as compounds which can lead to novel efficient drugs. Among them, 3-hydroxyscopoletin **2** is the most promising because of its high inhibitory potency (IC_{50} = 0.070 μM) compared with that of known active substances (28-31).

Since 4-hydroxycoumarin **11** caused no inhibition, the 5-lipoxygenase inhibition by the catechols **3** and **10** and the α-hydroxy-α,β-unsaturated lactones **2**, **5**, and **7** is assumed to proceed *via* chelation of the non-heme iron ion at the active center of the enzyme by these compounds, which consequently inhibit the enzyme redox reaction (28-31). Higher chelation and/or reduction

Table II. Increase of 5-Lipoxygenase[a] Inhibitory Activities of Coumarins **1**, **4**, **6**, and **8** through 3-Hydroxylation or Catechol Formation by a Cu^{2+} – Ascorbic Acid – O_2 System

	%, Inhibition at 25 μg/ml (IC_{50}, μM)[b]								
Coumarin			3-OH compd[c]			Catechol compd[d]			
Scopoletin	**1**	6	**2**	76	(4.3[a], 0.070[e])[b]	**3**	84	(1.9[a])[b]
Isoscopoletin	**4**	0	**5**	39		**3**	84	(1.9[a])[b]
Umbelliferone	**6**	0	**7**	65	(28[a])[b]	**3**	84	(1.9[a])[b]
4-Methyl-umbelliferone	**8**	2	----------			**10**	64	(1.3[a])[b]

a. The enzyme used is the 10,000×g supernatant from the centrifuged mixture of the homogenized rat basophilic leukemia cells. b. 50% inhibition concentration is designated in parentheses. c. 3-Hydroxylated compound. d. Catechol compound. e. The enzyme used is the 105,000×g supernatant.

Table III. Increase of α-D-Glucosidase Inhibitory Activities of Coumarins **1**, **4**, **6**, and **8** through 3-Hydroxylation or Catechol Formation by a Cu^{2+} – Ascorbic Acid – O_2 System

	%, Inhibition at 100 μg/ml (IC_{50}, μM)[a]								
Coumarin				3-OH compd[b]			Catechol compd[c]		
Scopoletin	**1**	60	(500)[a]	**2**	57	(380)[a]	**3**	54	(400)[a]
Isoscopoletin	**4**	0		**5**	38		**3**	54	(400)[a]
Umbelliferone	**6**	38		7	95	(17)[a]	**3**	54	(400)[a]
4-Methyl-umbelliferone	**8**	53	(530)[a]	----------			**10**	64	(310)[a]

a. 50% inhibitory concentration is designated in parentheses. b. 3-Hydroxylated compound. c. Catechol compound.

potencies to iron ion of the above compounds, esculetin **3**, 3-hydroxyscopoletin **2**, 3-hydroxyisoscopoletin **5**, 3-hydroxyumbelliferone **7**, and 4-methylesculetin **10** than those of *o*-methoxyphenols **1** and **4**, phenols **6** and **8**, and β-hydroxy-α,β-unsaturated lactone **11** can be presumed to result in the higher activities of the former compounds, **2**, **3**, **5**, **7**, and **10**.

Next, the α-D-glucosidase inhibitory activities of the above coumarins were assayed (Table III). *Via* 3-hydroxylation or catechol formation, all of the products were activated rather than their substrates. 4-Hydroxycoumarin **11** had low activity (33% inhibition at 100 μg/ml). 3-Hydroxyumbelliferone **7** shows promise as a lead compound for new drugs because of its high inhibitory activity (IC_{50} = 17 μM). These findings suggest that high inhibitory activity results when the catechol or α-hydroxy-α,β-unsaturated lactone structure is present in the molecule.

Regioselective hydroxylation of a coumarin ring *via* use of the Cu^{2+} – ascorbic acid – O_2 system in place of cytochrome P-450 resulted in the preparation of more bioactive derivatives; "metabolic activation" of coumarin compounds was reproduced by the biomimetic oxidation system *via* activation of molecular oxygen, which the enzyme carries out likewise.

The value of these reactions in bioorganic chemistry and biophysical chemistry has led us into further mechanistic studies which are currently in progress.

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