Evaluation of Synthesized Ester or Amide Coumarin Derivatives on Aromatase Inhibitory Activity

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Aromatase inhibitors are effective for the treatment of diseases such as breast cancer, which has led to an increase in their demand. However, only a limited number of aromatase inhibitor drugs are currently being marketed. In addition, considering the important aspect of drug resistance, the development of newer drug types is required. We have been developing inhibitors with backbone structures that differ from existing aromatase inhibitors. In this regard, we previously reported that diethylaminocoumarin dimers and thiazolyl coumarin derivatives possess strong aromatase inhibiting capabilities. In this study, we further examined the structure–activity relationships of coumarin derivatives synthesized from thiazolyl coumarin derivatives and their aromatase inhibiting capabilities. Consequently, amide coumarin N-benzhydryl-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (IC₅₀ values 4.5 µM) is inhibitor of aromatase. This inhibitor was found to be comparable aromatase inhibitory activity to the 1st generation aromatase inhibitor aminogluthethimide (3.2 µM). Substitution of the amide group on the amide coumarin derivative affects the aromatase inhibiting activity. Our findings suggest that the structure of each substituent changes the orientation of the compound in the active site of aromatase, thus creating a difference in their activities.

Key words breast cancer; aromatase; inhibitor; coumarin; amide; ester

INTRODUCTION

According to the GLOBOCAN 2018 database,¹ published by the International Agency for Research on Cancer (IARC), breast cancer is the most common cancer type among women globally. Women breast cancer accounts for 24.2% (2.1 million cases) of total new cancer cases, and the death rate is 15.0% (626679). The majority of breast cancers are estrogen-dependent tumors, and one-third of whole breast cancer cases and two-thirds of postmenopausal breast cancer cases are believed to be estrogen dependent. Women secrete estrogen from their ovaries or produce estrogen from androgens by the action of CYP19 (aromatase), which is a type of CYP. The estrogen regulation pathway changes after menopause. In premenopausal women, estrogen is primarily produced through the ovaries, whereas in postmenopausal women, it is produced from androgens by aromatase. Therefore, aromatase inhibitors (AIs) are quickly becoming the initial therapy of choice for postmenopausal women with estrogen-receptor-positive breast cancer. In addition, the use of AIs has been expanding from its target range. For example, AIs in combination with luteinizing hormone-releasing hormone (LH-RH) agonist has been shown to be effective for premenopausal breast cancer.² AIs also have been shown to be effective in treating endometrial cancer, endometritis, fibrosis uteri, fibroids, and uterine fibroids, which are highly estrogen dependent. Moreover, it has been reported that AIs are effective for infertility treatment because they stimulate the secretion of follicle-stimulating hormone.³ Given these findings, the demand for AIs has been increasing; however, currently available AIs are limited to three kinds: steroidal exemestane, anastrozole, and letrozole (a triazole derivative). Taking drug resistance and related adverse effects into consideration, AIs composed of different backbone structure types are required. Steroid metabolizing enzyme like an aromatase has a high substrate specificity; thus, most AIs currently being developed are either steroidal or triazole derivative types. Chen et al. reported that coumarin derivatives ¹,⁴, ²⁵ (Fig. 1) have the ability to inhibit aromatase, which led to the belief that coumarin derivatives may represent novel options for new breast cancer drugs.

Moreover, Ghosh et al. revealed the three dimensional structure of aromatase, which has a rigid active site that specifically recognizes androgens.⁶,⁷ Polar amino acid residues

Fig. 1. Structure of Coumarin Derivatives Expected to Be Anticancer Agents

Aromatase inhibitor: 1; 2; 17β-hydroxysteroid dehydrogenase inhibitor: 3; cancer cell proliferation inhibitors 4, 5, 2⁶,²⁵

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Many coumarin derivatives occur in nature, with more than 1300 different coumarin derivatives with plant origins, and if synthetic products are included, their number is immeasurable. Coumarin itself is known to have many medical benefits aside from being an anticoagulant, and possesses anti-inflammatory, antioxidant, antiviral, antibacterial, antihyperlipidemic, and anticancer properties. Zhou and colleagues reviewed over 200 coumarin derivatives and their applications. In addition, reviews published on coumarin and breast cancer treatments stated that various coumarins form structures similar to steroids in order to inhibit the activities of steroid hormones. In 2011, Gobec and colleagues developed 17β-hydroxysteroid dehydrogenase inhibitor which mimics estrone (Fig. 1). The authors found that the carbonyl group at 3-position of coumarin substitutes the carbonyl at 17-position of estrone to inhibit 17β-hydroxysteroid dehydrogenase. We have been studying the fluorescence property of a 7-diethylaminocoumarin analog with Ar group on position 3, the development of smoking cessation aids using the CYP2A6-inhibiting action of coumarin derivatives, and the development of tools to reveal the mechanisms underlying the drug interactions of coumarin and furanocoumarin based on the relationships between their structures and CYP3A4-inhibiting capabilities. In addition, we reported on the aromatase-inhibiting capability of a coumarin analog. In our previous study, we reported that the aromatase inhibiting capability of diethylaminocoumarin with a heterocycle on the 3-position changes depending on the position of the heteroatom (Figs. 3 Ia, Il). However, the synthesis of the coumarin derivative with a heterocycle requires multiple steps.
Moreover, it was reported that diethylaminocoumarin derivative 4 have antiproliferative activity toward T47D human breast cancer cell line. Lee et al. revealed 5a which has benzoxazole ring showed antiproliferative effect on a variety of different cancer cell lines (U87 MG: human glioma; B16: mouse melanoma; HeLa; human cervical carcinoma; DLD-1: human colorectal adenocarcinoma; SiHa: human cervical carcinoma; NIH 3T3: mouse embryonic fibroblast cell line; HUVEC: human umbilical vein endothelial cell). However, other coumarins without benzoxazole such as 5b, c did not show any cytotoxicity against these cell lines. These results suggested that coumarin compounds without benzoxazole may not show nonspecific cytotoxicity (Fig. 1). It is known that acylation can produce a large number of compounds easily, these derivatives are not expected to be nonspecific cytotoxicity; thus, we decided to mimic the heterocycle with an ester or amide bond. For this we designed ester derivative 6 and amide derivative 7 as derivatives that have heteroatoms on the positions relative to 1a Het and reverse amide 8 that corresponds to 1a. For comparison, we also synthesized compound 9 (Fig. 4). The ester form can take conformations similar to 1b and 1c and the amide can take conformations similar to 1d and 1e. However, it is assumed that when R is bulky, steric repulsion is predicted to occur within the pocket and the compounds take conformations with the alkyl groups on the outside of the pocket. This is expected to result in greater affinity with hydrophobic residues in the access channel, as was the case for derivatives reported by Ghosh et al. Thus, we decided to synthesize derivatives 6a–d and 7a–f with various substituents on R. For comparison, we added similar substituents on the reverse amide (8a–e). Therefore, the purpose of this study was to synthesize diethylaminocoumarin derivatives 6–9, measure their aromatase inhibiting capabilities, and reveal their structure–activity relationships.

MATERIALS AND METHODS

Chemistry NMR spectra measured in CDCl3 or dimethyl sulfoxide (DMSO)-d6 at 500 MHz by using JEOL JNM-ECA 500 spectrometers. The chemical shifts are reported in ppm (δ) relative to TMS (0.0 ppm) as the internal standard. Coupling constants (J) were expressed in hertz (Hz) and spin multiplicities were given as s (singlet), d (doublet), dd (double doublet), t (triplet), dt (double triplet), q (quartet), br (broad). TLC was performed with Merck 60 F254 silica gel plate. Column chromatography was conducted using Silica Gel 60 (Merck, 63–200 mesh) or Silica Gel 60 N (Kanto Kagaku, 100–210 mesh). Mass spectra were obtained on a JEOL Accu TOF LC-plus. High resolution (HR)-MS were analyzed using reserpine as the internal standard.

Ethyl 7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylate (11)

A solution of 4-(diethylamino)salicylaldehyde (1.9 g, 10 mmol), diethyl malonate (5.8 g, 36 mmol), piperidine (0.4 g, 5 mmol) in ethanol (8.5 mL) was refluxed for 4.5 h. The reaction mixture was cooled at room temperature, and solvent was removed under vacuo. The product was purified by silica gel column chromatography (hexane : ethyl acetate = 2:1), to give 11 (2.6 g, 90%).

1H-NMR (CDCl3) δ: 1.21 (6H, t, J = 7.5 Hz), 1.37 (3H, t, J = 6.9 Hz), 3.42 (4H, q, J = 7.5 Hz), 4.35 (2H, q, J = 6.9 Hz), 6.43 (1H, d, J = 2.9 Hz), 6.58 (1H, dd, J = 2.9, 8.6 Hz), 7.33 (1H, d, J = 8.6 Hz), 8.40 (1H, s); 13C-NMR (CDCl3) δ: 12.5, 14.5, 45.2, 61.2, 97.0, 105.8, 108.7, 111.0, 149.3, 152.9, 158.4, 158.5, 164.3; electrospray ionization (ESI)-HR-MS m/z: 312.1215 (Calcd for C16H19NNaO4; 312.1212); ESI-low resolution (LR)-MS m/z: 312 (MNa+).

7-(Diethylamino)-2-oxo-2H-chromene-3-carboxylic Acid (12)

A solution of 11 (2.9 g, 10 mmol), KOH (5.6 g, 100 mmol) in water (48 mL), and ethanol (12 mL) was refluxed for 30 min. The reaction mixture was cooled at 0°C, HCl was added at pH 7.0. The product was purified by recrystallization from ethanol, to give 12 (2.5 g, 96%).

1H-NMR (CDCl3) δ: 1.26 (6H, t, J = 6.9 Hz), 3.49 (4H, q, J = 6.9 Hz), 6.53 (1H, d, J = 2.3 Hz), 6.70 (1H, dd, J = 2.3, 9.2 Hz), 7.45 (1H, d, J = 9.2 Hz), 8.65 (1H, s), 12.33 (1H, s); 13C-NMR (CDCl3) δ: 12.5, 45.4, 97.0, 105.8, 108.7, 111.0, 132.0, 150.4, 153.8, 158.2, 164.5, 166.6; ESI-HR-MS m/z: 284.0913 (Calcd for C14H15NNaO4; 284.0899); ESI-LR-MS
8.0 Hz, 6H, –OCH3), 6.49 (1H, d, J = 6.4 Hz, –OCH3), 6.49 (1H, d, J = 9.2 Hz, 6-H), 6.92 (2H, d, J = 9.2 Hz, 2'-H), 7.62 (2H, d, J = 9.2 Hz, 3'-H), 7.72 (1H, d, J = 9.2 Hz, 5-H), 8.75 (1H, s, 4-H), 10.59 (1H, brs, NH); 13C-NMR (CDCl3) δ: 12.4, 45.0, 55.4, 96.5, 108.5, 110.0, 114.0, 121.8, 131.1, 131.5, 148.1, 152.6, 156.1, 157.6, 160.7, 163.0; ESI-HR-MS m/z: 389.1477 (Calcd for C21H17N2NaO3: 389.1477); ESI-LR-MS m/z: 389 (MNa+).

7-(Diethylamino)-2-oxo-2H-chromene-3-carboxamide (7b)
Yield: 85%; 1H-NMR (DMSO-d6) δ: 1.15 (6H, t, J = 6.9 Hz, –CH2CH3), 3.50 (4H, q, J = 6.9 Hz, –CH2CH3), 3.89 (3H, s, OCH3), 6.49 (1H, d, J = 2.3 Hz, 8-H), 6.84 (1H, dd, J = 2.3, 9.2 Hz, 6-H), 6.95 (1H, m, 3'-H), 7.06–7.07 (2H, m, 4', 5'-H), 7.73 (1H, d, J = 9.1 Hz, 5-H), 8.45 (1H, d, J = 8.1 Hz, 6'-H), 8.78 (1H, s, 4-H), 11.13 (1H, brs, NH); 13C-NMR (CDCl3) δ: 12.5, 45.2, 56.1, 96.7, 108.7, 110.1, 110.2, 111.0, 120.6, 121.0, 123.9, 128.4, 131.3, 148.3, 149.1, 152.8, 157.9, 161.0, 162.9; ESI-HR-MS m/z: 389.1436 (Calcd for C21H17N2NaO3: 389.1477); ESI-LR-MS m/z: 389 (MNa+).

N-Benzyl-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (7c)
Yield: 43%; 1H-NMR (DMSO-d6) δ: 1.13 (6H, t, J = 6.9 Hz, –CH2CH3), 3.47 (4H, q, J = 6.9 Hz, –CH2CH3), 4.51 (2H, d, J = 5.7 Hz, methylene), 6.61 (1H, d, J = 2.3 Hz, 8-H), 6.80 (1H, dd, J = 2.3, 9.2 Hz, 6-H), 7.24 (1H, m, 4'-H), 7.31–7.32 (4H, m, 2', 3'-H), 7.67 (1H, d, J = 9.2 Hz, 5-H), 8.67 (1H, s, 4-H), 9.02 (1H, t, J = 5.7 Hz, NH); 13C-NMR (CDCl3) δ: 12.5, 43.7, 45.2, 96.7, 108.5, 110.0, 110.3, 127.2, 127.8, 128.7, 131.2, 133.8, 148.4, 152.7, 157.8, 162.8, 163.3; ESI-HR-MS m/z: 373.1519 (Calcd for C21H17N2NaO3: 373.1528); ESI-LR-MS m/z: 373 (MNa+).

7-(Diethylamino)-2-oxo-N-(1-phenylethyl)-2H-chromene-3-carboxamide (7d)
Yield: 31%; 1H-NMR (DMSO-d6) δ: 1.13 (6H, t, J = 6.9 Hz, –CH2CH3), 1.47 (3H, d, J = 6.9 Hz, CH3), 3.47 (4H, q, J = 6.9 Hz, –CH2CH3), 5.11 (1H, m, methylene), 6.62 (1H, d, J = 2.3 Hz, 8-H), 6.79 (1H, dd, J = 2.3, 9.2 Hz, 6-H), 7.24 (1H, m, 4'-H), 7.32–7.37 (4H, m, 2', 3'-H), 7.66 (1H, d, J = 9.2 Hz, 5-H), 8.63 (1H, s, 4-H), 8.97 (1H, d, J = 8.0 Hz, NH); 13C-NMR (CDCl3) δ: 12.4, 22.6, 45.0, 49.0, 96.5, 108.3, 109.8, 110.3, 126.1, 127.0, 128.5, 131.1, 143.7, 148.1, 152.4, 157.6, 162.2, 162.8; ESI-HR-MS m/z: 387.1690 (Calcd for C22H20N2NaO3: 387.1685); ESI-LR-MS m/z: 387 (MNa+).

N-Benzhydryl-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (7e)
Yield: 51%; 1H-NMR (DMSO-d6) δ: 1.13 (6H, t, J = 6.9 Hz, –CH2CH3), 3.48 (4H, q, J = 6.9 Hz, –CH2CH3), 6.25 (1H, d, J = 8.0 Hz, methylene), 6.63 (1H, dd, J = 2.3, 9.2 Hz, 6-H), 7.24–7.27 (2H, m, 4'-H), 7.31–7.36 (8H, m, 2', 3'-H), 7.68 (1H, d, J = 9.2 Hz, 5-H), 8.65 (1H, s, 4'-H), 9.47 (1H, d, J = 8.0 Hz, NH); 13C-NMR (CDCl3) δ: 12.4, 45.0, 57.1, 96.5, 108.3, 109.9, 110.1, 127.2, 127.4, 128.6, 131.1, 141.9, 148.3, 152.5, 157.6, 162.3, 162.8; ESI-HR-MS m/z: 449.1844 (Calcd for C22H20N2NaO3: 449.1844); ESI-LR-MS m/z: 449 (MNa+).

7-(Diethylamino)-7-N-(4-methoxybenzyl)-2-oxo-2H-chromene-3-carboxamide (7f)
Yield: 60%; 1H-NMR (DMSO-d6) δ: 1.13 (6H, t, J = 6.9 Hz, –CH2CH3), 3.47 (4H, q, J = 6.9 Hz, –CH2CH3), 3.72 (3H, s, OCH3), 4.42 (2H, d, J = 5.7 Hz, methylene), 6.60 (1H, d,
(1H, d, J = 6.9 Hz, 5-H), 6.72 (1H, d, J = 9.2 Hz, 5-H), 7.21 (1H, t, J = 7.4 Hz, 2-H), 7.37–7.40 (3H, m, 3, 5, 7-H), 8.41 (1H, s, 4'-H), 9.46 (1H, brs, NH); 13C-NMR (CDCl 3) δ: 12.5, 18.6, 44.8, 48.3, 97.5, 108.3, 109.7, 119.0, 125.5, 127.6, 127.8, 129.2, 140.6, 149.5, 152.7, 159.6, 173.1; ESI-HER-MS m/z: 387.1685 (Calcd for C14H14N2NaO4: 387.1685); ESI-LR-MS m/z: 387 (MNa+).

9-(7-(Diethylamino)-2-oxo-2H-chromen-3-yl)-2,2-diphenylenopramidine (8e)

Yield: 92%; 1H-NMR (DMSO-d6) δ: 1.09 (6H, t, J = 6.9 Hz, -CH2CH3), 3.39 (4H, q, J = 6.9 Hz, -CH2CH3), 5.58 (1H, s, methylene), 6.52 (1H, d, J = 2.3 Hz, 8'-H), 6.68 (1H, dd, J = 2.3, 9.2 Hz, 6'-H), 7.22–7.62 (2H, m, 4-H), 7.31–7.37 (7H, m, 2-H, 3-H, 4-H), 7.41 (1H, d, J = 9.2 Hz, 5'-H), 8.84 (1H, s, 4'-H), 9.83 (1H, brs, NH); 13C-NMR (CDCl 3) δ: 12.5, 44.8, 60.1, 97.5, 108.3, 109.7, 118.9, 125.9, 127.6, 128.8, 128.9, 129.0, 138.8, 149.6, 152.8, 159.5, 170.9; ESI-HER-MS m/z: 449.1829 (Calcd for C25H22N2NaO4: 449.1841); ESI-LR-MS m/z: 449 (MNa+).

N-(7-(Diethylamino)-2-oxo-2H-chromen-3-yl)-2-(4-methoxyphenyl)acetamide (8d)

Yield: 54%; 1H-NMR (DMSO-d6) δ: 1.10 (6H, t, J = 6.9 Hz, -CH2CH3), 3.39 (4H, q, J = 6.9 Hz, -CH2CH3), 3.67 (2H, s, methylene), 3.72 (3H, s, OCH3), 6.52 (1H, d, J = 2.3 Hz, 8'-H), 6.67 (1H, dd, J = 2.3, 9.2 Hz, 6'-H), 6.87 (2H, d, J = 8.6 Hz, 3-H), 7.24 (2H, d, J = 8.6 Hz, 2-H), 7.38 (1H, d, J = 9.2 Hz, 5'-H), 8.39 (1H, s, 4'-H), 9.48 (1H, brs, NH); 13C-NMR (CDCl 3) δ: 12.5, 44.8, 44.8, 55.4, 97.5, 108.3, 109.7, 114.7, 119.0, 125.6, 126.0, 128.8, 130.5, 149.5, 152.8, 159.1, 170.3; ESI-HER-MS m/z: 403.1662 (Calcd for C24H20N2O4: 403.1634); ESI-LR-MS m/z: 403 (MNa+).

7-(Diethylamino)-3-(4-(methoxybenzyl)2H)-chromen-2-one (9)

A solution of 14 (232 mg, 1 mmol), 4-methoxybenzyl chloride (155 mg, 1 mmol), K2CO3 (414 mg, 3 mmol) in DMF (10 mL), H2O (3 mL) was refluxed for 2 d. The reaction mixture was cooled at room temperature. To the reaction mixture, HCl was added at pH 7.0. The mixture was partitioned between water (10 mL) and ethyl acetate (30 mL). The organic phase was washed with water (20 mL*2), brine (20 mL), dried over Na2SO4, and solvent was removed under vacuo. The product was purified by silica gel column chromatography (hexane:ethyl acetate = 3:1), to give 9 (71 mg, 20%).

1H-NMR (CDCl 3) δ: 1.14 (6H, t, J = 6.9 Hz, -CH2CH3), 3.33 (4H, q, J = 6.9 Hz, -CH2CH3), 3.77 (3H, s, OCH3), 4.22 (2H, s, methylene), 4.81 (1H, brs, NH), 6.28 (1H, s, 4-H), 6.49 (1H, d, J = 2.3 Hz, 8-H), 6.53 (1H, dd, J = 2.3, 9.2 Hz, 6-H), 6.85 (2H, d, J = 8.6 Hz, 3-H), 7.06 (1H, d, J = 9.2 Hz, 5-H), 7.23–7.26 (2H, m, 2'-H); 13C-NMR (CDCl 3) δ: 12.5, 45.0, 47.3, 55.3, 98.1, 108.4, 109.4, 110.2, 114.0, 125.9, 128.6, 129.3, 129.9, 146.8, 150.3, 158.9, 160.4; ESI-HER-MS m/z: 375.1663 (Calcd for C24H20N2NaO4: 373.1528); ESI-LR-MS m/z: 373 (MNa+).
for C_{21}H_{24}N_{2}NaO_{3}: 375.1685); ESI-LR-MS m/z: 375 (MNa\(^+\)).

**Biological Evaluation**

**In Vitro Aromatase Inhibitory Activity**

[1\(^{3}H\)]-Androstenedione aromatization (tritirated water release assay)

The aromatization of [1\(^{3}H\)]-androstenedione was used as the index of aromatase activity. The rate of [1\(^{3}H\)]-androstenedione aromatization was determined by the method of Thompson and Siiteri.\(^{22}\) Expressed human CYP19A1 and CYP3A4 (2.5 pmol) were incubated with androstenedione (final concentration 0.2 \(\mu\)M) and inhibitor (0.05–20 \(\mu\)M) at 37°C in the presence of phosphate buffer (100 mM, pH 7.4), NADP (0.8 mM), glucose-6-phosphate (7.9 mM), and glucose-6-phosphate dehydrogenase (1 unit/\(\mu\)L), MgCl\(_2\) (5.9 mM) in total volume of 500 \(\mu\)L. The reaction was started by addition of the NADPH at 37°C. Reactions were stopped after 15 min incubation with 500 \(\mu\)L of CHCl\(_3\). After further mixing, centrifuge for 5 min at 27000rpm. Supernatant were analyzed by liquid scintillation counter ALOKA LSC-6100.

**In Vitro CYP3A4 Inhibitory Activity**

Testosterone 6\(^{\beta}\)-Hydroxylation

The 6\(^{\beta}\)-hydroxylation of testosterone was used as the index of CYP3A4 activity. The rate of testosterone 6\(^{\beta}\)-hydroxylation was determined by the method of Guo et al.\(^{23}\) Expressed human CYP3A4 (2.5 pmol) were incubated with testosterone (final concentration 0.2 \(\mu\)M) and inhibitor (5, 10 \(\mu\)M) at 37°C in the presence of phosphate buffer (100 mM, pH 7.4), NADP (0.8 mM), glucose-6-phosphate (7.9 mM), and glucose-6-phosphate dehydrogenase (1 unit/\(\mu\)L), MgCl\(_2\) (5.9 mM) in total volume of 500 \(\mu\)L. There was a 5 min preincubation step at 37°C before the reaction was started by addition of the NADPH generating system. Reactions were stopped after 15 min incubation with 3 mL of AcOEt. After 11\(^{\alpha}\)-hydroxy progesterone (internal standard) was added, centrifuge for 5 min at 2800 rpm. Supernatant were analyzed by HPLC-fluorescence method (Ex 280 nm, Em 310 nm). The HPLC system consisted of a Hitachi model L7100 pump, Hitachi D-7500 detector, and Waters 2475 multi \(\lambda\) fluorescence detector.

**RESULTS AND DISCUSSION**

**Synthesis**

Coumarin derivatives synthesized by a conventional method (Chart 1). 3-Carboxylicoumarin analog 12, prepared by the hydrolysis of 11, was allowed to react with an alcohol or amine to form ester derivative 6 or amide derivative 7. Compound 13 was reduced to form 3-aminocoumarine derivative 14. Condensing 14 with an acyl chloride formed reverse amide 18. Condensation of 14 and an alkyl halide formed compound 9. We confirmed amide proton by means of heavy water addition. Amide 7 proton were slow exchange with heavy water, however reverse amide 8 proton were fast.

**Aromatase Inhibitory Activity Assay**

CYP19 (aromatase) metabolizes the A-ring of androstenedione onto the aromatised estrone. Because one molecule of formic acid and one molecule of water are released simultaneously, a \(^3\)H\(_2\)O release assay and a H\(^{14}\)COOH release assay, using [1\(^{\beta}\)-\(^3\)H]-androstenedione and [19\(^{14}\)C]-androstenedione as substrates, are used to measure aromatase activity.\(^{22}\) Herein, aromatase activity was measured in a similar way by using the metabolic reaction of [1\(^{\beta}\)-\(^3\)H]-androstenedione.\(^{23}\) Figure 5 shows the percentage of control is indicated for 10 \(\mu\)M of coumarin derivatives 6–9 and positive control aminoglutethimide (AG, Fig. 4). The value of IC\(_{50}\) was calculated by probit analyses (Table 1). The first-generation AI, AG has IC\(_{50}\) values 3.2 \(\mu\)M as same as O-demethylation was determined by the method of Madeira et al.\(^{24}\) Expressed human CYP2D6 (2 pmol) were incubated with dextromethorphan (final concentration 4 \(\mu\)M) and inhibitor (5, 10 \(\mu\)M) at 37°C in the presence of phosphate buffer (100 mM, pH 7.4), NADP (0.8 mM), glucose-6-phosphate (7.9 mM), and glucose-6-phosphate dehydrogenase (1 unit/\(\mu\)L), MgCl\(_2\) (5.9 mM) in total volume of 500 \(\mu\)L. The reaction was started by addition of the NADPH generating system, and after incubated for 10 min. After incubation, the reaction was terminated by addition of 50 \(\mu\)L of 60% HClO\(_4\). After further mixing, centrifuge for 10 min at 10000 rpm, 4°C. Supernatant were analyzed by HPLC-fluorescence method (Ex 280 nm, Em 310 nm). The HPLC system consisted of a Hitachi model L7100 pump, Hitachi D-7500 detector, and Waters 2475 multi \(\lambda\) fluorescence detector.

**Chart 1.**

Reagents and conditions. i) diethyl malonate, piperidine, EtOH, reflux. ii) KOH, H\(_2\)O, EtOH, reflux. iii) ROH or RNH\(_2\), isobutyl chloroformate, Et\(_3\)N, THF, 50°C. iv) ethyl nitroacetate, piperidine, AcOH, BuOH, reflux. v) SnCl\(_2\), HCl, H\(_2\)O, room temperature (r.t.). vi) RCOCl, piperidine, r.t. vii) 4-methoxybenzylchloride, K\(_2\)CO\(_3\), DMF, H\(_2\)O, reflux.
The percentage of control of ester derivative 6 toward aromatase was 79–99%; thus, its inhibiting capability was low. 4-methoxy form 6a, 4-nitro form 6b, and 4-bromo form 6c having different substituent effects and 2-methoxy form having the substituent at a different position did not exhibit significant differences in their activities. Between the compound 6a and 7a pair or the 6d and 7b pair, having the same aryl group in each pair, amide derivative 7 exhibited stronger inhibiting effects. This indicates that ester derivatives have lower potentials as AIs. In contrast, the amide derivatives 7 showed different activities depending on their aryl group structures (41–83%). Based on the observation that compound 9, which does not have a carbonyl group, did not inhibit aromatase, it appears that the amide structure is essential for the synthesized derivatives. The inhibiting capability of amide 7f was stronger than that of 7a, whereas that of reverse amide 8e was stronger than that of 8a. This demonstrates the effectiveness of having a methylene between the aryl group and amide, which provides a flexible conformation for the substituents. When adding a substituent to methylene, amide series compounds 7 having a bulkier substituent exhibited increased inhibiting capabilities. Meanwhile, reverse amide series compounds 8 exhibited the opposite effect. Considering the influences of the structural conformation mentioned in the introduction, it can be assumed that the amide forms the confirmation shown in Fig. 6 III and the reverse amide forms the confirmation shown in Fig. 6 VI. The R1 and R2 of the amide 7 are positioned at the location relative to the steroid A ring and access channel, respectively. When either of the alkyl groups is bulky, the affinity between it and the active site or access channel increases. The bulkiness of the substituent groups and their activities may have a direct relationship. Conversely, the alkyl group on the reverse amide 8 is positioned in the proximity of heme of the active site. In addition, if compound 8 has an s-trans conformation, steric repulsion would occur between the alkyl groups in the reverse amide portion and the active site of amino acids. Thus, when the substituent group is bulky, it creates steric repulsion in the molecule or between the functional group and the active site. So, the bulkiness of the substituent group and their activities may have reverse relationships.

Docking studies of human aromatase CYP19A1 (PDB ID: 3S7S)7) with synthesized coumarin derivatives were performed by Molegro Virtual Docker ver10, which is a protein–ligand docking simulation program (data not shown). No parameters were found to correlate either with the aromatase inhibitory activity between the important interacting amino acid at the active site and the orientations of our coumarin derivatives or with any intermolecular hydrogen bonds. Another possibility is that in comparison with compound 8, compound 7 has a stronger intramolecular hydrogen bond between the amide

![Fig. 6. Estimated Structures of Amide Coumarin 7 (III), and Reverse Amide Coumarin 8 (IV) in Aromatase Active Site](image)

Table 1. IC50 Values of Coumarin Derivatives and AG on [1β-3H] Androstenedione Aromatization

| Compounds | IC50 (µM) |
|-----------|-----------|
| 7b        | 11.85     |
| 7d        | 10.72     |
| 7e        | 4.52      |
| 8e        | 11.48     |
| AG        | 3.20      |

Fig. 5. Inhibitory Effects of Coumarin Derivatives on [1β-3H] Androstenedione Aromatization

Coumarin derivatives and AG were assayed at 10µM. Each column represents mean ± S.D. (n = 3).
proton and the coumarin carbonyl oxygen, thus affecting the inhibitory activity. But no correlation was found between the inhibitory activity and hydrogen bond. The active site of aromatase is rigid; however, the access channel of aromatase is flexible as it rests on the lipid bilayer.\(^2\) Ghosh et al. designed steroid analogs based on the access channel and produced crystals from the aromatase-designed analog complexes.\(^3\) They reported that the binding affinity between the access channel amino acids with a steroid \(\beta\) was found to act as effectively as the existing aromatase inhibitor AG. Aromatase is an enzyme that converts androgens to oestrogens. The majority of currently developed aromatase inhibitors have comparatively high substrate specificity to CYP molecular species that comprise steroid derivatives or anastrozole- and letrozole-like triazole derivatives. Coumarin derivatives identified in the present study are materials that, in addition to enabling new possibilities for coumarin derivatives, paving the way for further development of aromatase inhibitors on the basis of structure–activity relationship studies that use these coumarins as a seed.

**CONCLUSION**

The aim of this work was to synthesize coumarin derivatives (ester, amide, reverse amide, and other type) as a view to determining the relationship between the structure of the inhibitors and their CYP19 (aromatase) inhibitory activity. The amide group on the amide coumarin derivative affects the aromatase inhibiting activity, and our findings suggest that the structure of each substituent changes the orientation of the compound in the active site of aromatase, thus creating a difference in their activities.

\(N\)-Benzhydryl-7-(diethylamino)-2-oxo-2\(H\)-chromene-3-carboxamide was found to act as effectively as the existing aromatase inhibitor AG. Aromatase is an enzyme that converts androgens to oestrogens. The majority of currently developed aromatase inhibitors have comparatively high substrate specificity to CYP molecular species that comprise steroid derivatives or anastrozole- and letrozole-like triazole derivatives. Coumarin derivatives identified in the present study are materials that, in addition to enabling new possibilities for coumarin derivatives, paving the way for further development of aromatase inhibitors on the basis of structure–activity relationship studies that use these compounds as a seed.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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