Discovery of a Novel Acyl-CoA: Cholesterol Acyltransferase Inhibitor: The Synthesis, Biological Evaluation, and Reduced Adrenal Toxicity of (4-Phenylcoumarin)acetanilide Derivatives with a Carboxylic Acid Moiety

Masaki Ogino,* Yoshihisa Nakada, Nobuyuki Negoro, Shigekazu Itokawa, Satoshi Nishimura, Tsukasa Sanada, Tomoko Satomi, Shunbun Kita, Kazuki Kubo, and Shogo Marui

Pharmaceutical Research Division, Takeda Pharmaceutical Company, Ltd.; 2–26–1 Muraoka-higashi, Fujisawa, Kanagawa 251–8555, Japan. Received July 28, 2011; accepted August 16, 2011; published online August 17, 2011

As a part of our research for novel potent and orally available acyl-CoA: cholesterol acyltransferase (ACAT) inhibitors that can be used as anti-atherosclerotic agents, we recently reported the discovery of the (4-phenylcoumarine)acetanilide derivative 1. However, compound 1 showed adrenal toxicity in animal models. In order to search for safer ACAT inhibitors that do not have adrenal toxicity, we examined the inhibitory activity of ACAT in human macrophage and adrenal cells. The introduction of a carboxylic acid moiety on the pendant phenyl ring and the adjustment of the lipophilicity led to the discovery of (2*E*)-3-[7-chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acrylic acid (21e), which showed potent ACAT inhibitory activity in macrophages and a selectivity of around 30-fold over adrenal cells. In addition, compound 21e showed high adrenal safety in guinea pigs.

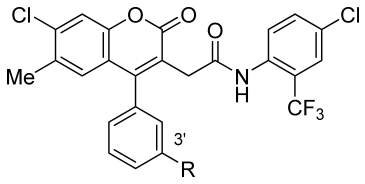
Key words acyl-CoA: cholesterol acyltransferase inhibitor; cholesterol; atherosclerosis; adrenal toxicity; macrophage; (4-phenylcoumarin)acetanilide

Atherosclerosis is one of the risk factors for stroke and coronary heart diseases. The progression of atherosclerotic lesions is associated with the accumulation of cholesteryl esters in macrophage-derived foam cells of the arterial wall.[1)]

Acyl-CoA: cholesterol acyltransferase (ACAT) is an intracellular enzyme that catalyzes the intracellular cholesterol esterification. Two ACAT isozymes have been identified to date. ACAT-1 is expressed ubiquitously in various human tissues and cells, such as various types of macrophages, adrenal glands, sebaceous glands, liver, and intestine.[2—5)] ACAT-2 is expressed exclusively in the small intestine and liver.[2—5)] The progression of atherosclerotic lesions is associated with the accumulation of cholesteryl esters through ACAT-1 in macrophage foam cells of the arterial wall.[5—7)]

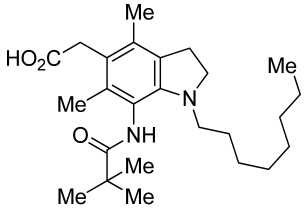
Approaches that target arterial plaques with ACAT inhibitors have been investigated experimentally for two decades.[8)] ACAT inhibitors interfere with intracellular cholesterol transport within plaque macrophages and may delay the formation of foam cells while activating reverse cholesterol transport. Experimental evidence suggested that ACAT inhibitors reduced and stabilized atherosclerotic plaques in animals.[9—11)] In addition, we have previously reported that the (4-phenylcoumarin)acetanilide derivative **1** is a potent ACAT inhibitor, which showed potent regressive effects on atherosclerotic plaques in apolipoprotein E (apoE)-knockout mice[12)] (Table 1). However, compound **1** and its related compounds showed potent adrenal toxicity in guinea pigs (data not shown).

Table 1. ACAT Inhibitory Activity of Compounds **1** and **2**



Compds.	R	Enzyme assay[a)] IC_{50} (nM)	Cell-based assay[b)] IC_{50} (nM)		
			Macrophage	Adrenal	Selectivity[c)]
1	–Cl	12	5	14	3
2	$-CO_2H$	125	96	560	6

a) Enzymatic ACAT inhibitory activity was determined by using homogenates from human THP-1 macrophages. *b*) Cellar ACAT inhibitory activity in human macrophage and adrenal was determined by using THP-1 and H295R cell-lines, respectively. *c*) Selectivity was evaluated by the value of IC_{50} (adrenal)/IC_{50} (macrophage).



Pactimibe

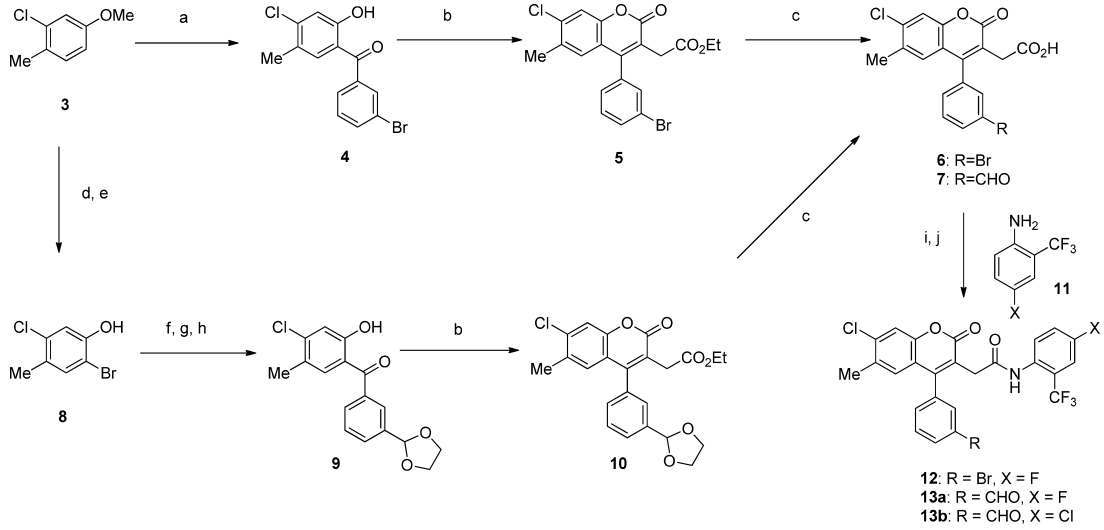
Fig. 1. Structure of Pactimibe

Although the adrenal toxicity was probably caused by the acute increase in free cholesterol levels in adrenal cells, the relationship between ACAT inhibition and adrenal toxicity has not been fully clarified.[13—15)] Recently, Takahashi *et al.* reported that pactimibe showed less potent ACAT inhibitory activity in adrenal tissue than in other tissues[16)] (Fig. 1). While the cause was not discussed in the paper, we speculated that it is possible to find compounds possessing a similar profile by using cell-based assays and that such compounds may show reduced adrenal toxicity. By the screening of our compound collection around compound **1**, compound **2** was found to show moderate selectivity for macrophages over adrenal cells in ACAT inhibition (Table 1). The common feature of both **2** and pactimibe seems to be the carboxylic acid moiety, and we therefore hypothesized that this acidic moiety could lead to the identification of selective compounds. In this paper, we report a modification of the carboxylic acid moiety of compound **2** in order to improve

* To whom correspondence should be addressed. e-mail: Ogino_Masaki@takeda.co.jp

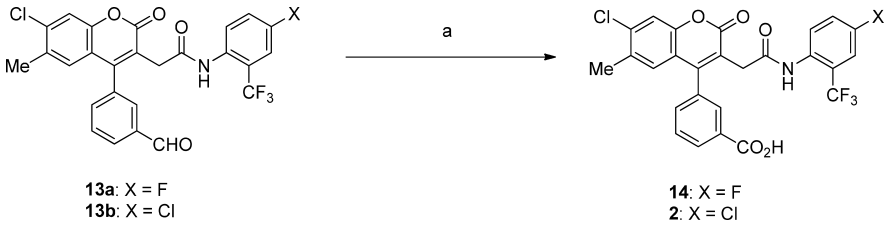
the potency and the selectivity for macrophages in cell-based assays. The adrenal toxicity of the optimized compounds was also evaluated.

Chemistry (4-Phenylcoumarin)acetanilide derivatives were synthesized by the construction of the coumarin skeleton according to our previous report (Chart 1)[12] and the successive introduction of a carboxylic acid moiety (Charts 2—6). 2-Hydroxybenzophenone **4**, which was prepared from anisole **3** and 3-bromobenzoyl chloride by Friedel–Crafts acylation, was converted into coumarinacetate **5** by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)-mediated condensation with ethyl 4-chloro-4-oxobutanoate and cyclization in one-pot synthesis. The ester **5** was hydrolyzed under acidic conditions to give acetic acid **6**. Aldehyde **7** was prepared by another method. Anisole **3** was transformed into phenol **8** by bromination and demethylation. Treatment of compound **8** with *n*-butyl lithium generated a dilithiated compound, which reacted with 3-(1,3-dioxolan-2-yl)benzaldehyde, followed by oxidation with manganese(IV) dioxide to afford 2-hydroxybenzophenone **9**. Construction of the coumarin ring was performed by the typical method to give coumarinacetate **10**. Hydrolysis of both the ester group and the 1,3-dioxolan group under acidic conditions resulted in coumarinacetic acid **7**. The acids **6** and **7** was converted into acyl chloride,



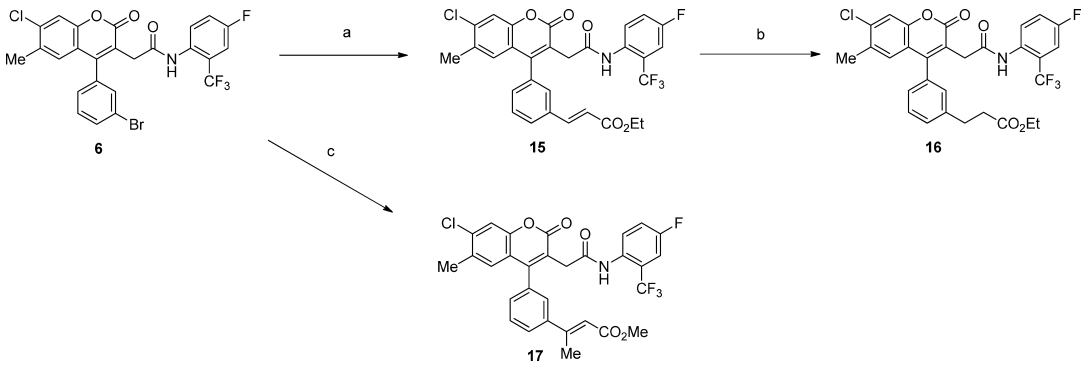
Reagents and conditions: (a) 3-bromobenzoyl chloride, $AlCl_3$, chlorobenzene, 100 °C, 0.5 h; (b) $EtOCOCH_2CH_2COCl$, DBU, CH_3CN, 40 °C, 0.5 h; (c) HCl, AcOH, reflux, 1 h; (d) Br_2, AcOH, rt, 1 h; (e) BBr_3, CH_2Cl_2, −78 °C to rt, 2 h; (f) *n*-BuLi in hexane, THF, −78 °C, 0.5 h; (g) 3-(1,3-dioxolan-2-yl)benzaldehyde, THF, −78 °C, 1 h; (h) MnO_2, toluene, reflux, 4 h; (i) $(COCl)_2$, DMF (cat.), THF, rt, 0.5 h; (j) **11**, THF, rt, overnight.

Chart 1



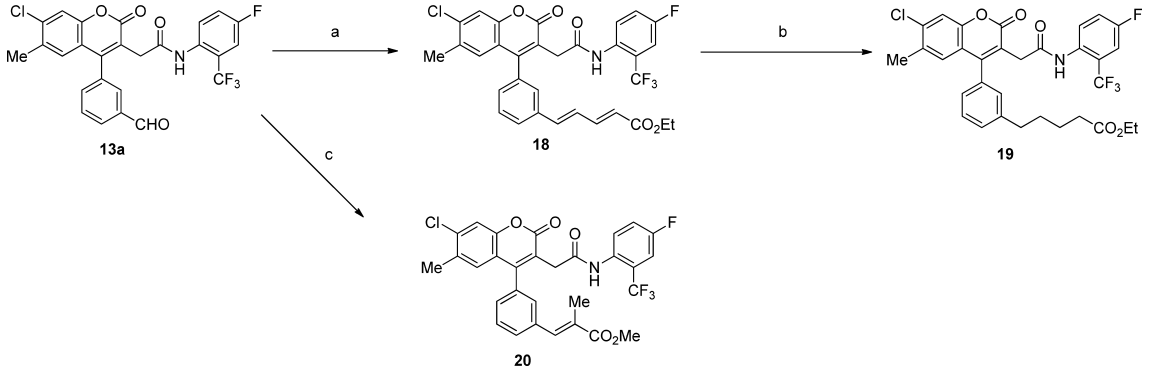
Reagents and conditions: (a) $NaClO_2$, NaH_2PO_4, ^{t}BuOH, 2-methyl-2-butene, THF, water, rt, 2 h.

Chart 2



Reagents and conditions: (a) CH_2=$CHCO_2Et$, $Pd(OAc)_2$, Ph_3P, Et_3N, DMF, 100 °C, 5 h; (b) Raney Ni, H_2, EtOH, rt, overnight; (c) methyl crotonate, $Pd(OAc)_2$, Ph_3P, Et_3N, DMF, 120 °C, 5 h.

Chart 3

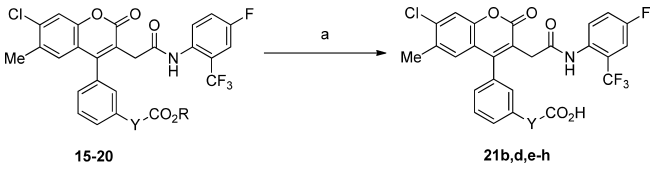


Reagents and conditions: (a) triethyl 4-phosphonocrotonate, NaH, THF, 0 °C to rt, 0.5 h; (b) Raney Ni, H_2, EtOH, rt, overnigh; (c) triethyl 2-phosphonopropionate, NaH, THF, 0 °C to rt 0.5 h.

Chart 4

and the corresponding acyl chloride was coupled with anilines **11** to afford coumarinacetanilides **12** and **13a**, **b**.

The aldehydes **13a** and **b** were oxidized to give corresponding acids **2** and **14** as shown in Chart 2. The bromide **6** was converted into propenoate **15** or its β-methyl analog **17** by the Heck reaction with ethyl acrylate or methyl crotonate (Chart 3). The propenoate **15** was transformed into propionate **16** by hydrogenation with Raney Ni. The aldehyde **13a** was reacted with triethyl 4-phosphonopropionate and triethyl 2-phosphonocrotonate to give diene **18** and α-methyl analog **20** (Chart 4). The diene **18** was converted into pentanoate **19** by hydrogenation. These esters **15—20** were hydrolyzed to give the corresponding acids **21b**, **d**, and **e**—**h** (Chart 5). Acetic acid **21a** and butyric acid **21c** were synthesized by the Erundt-ainstert reaction as a key step (Chart 6).[17] The acids **14** and **21b** were converted into acyl chloride, and the corresponding acyl chloride was reacted with diazomethane, which was followed by treatment with methanol and silver(II) oxide to afford methyl ester analogs **22** and **23**, respectively. Hydrolysis of the esters **22** and **23** afforded corresponding acids **21a** and **c**.



	–Y–CO_2H
21b	–$CH_2CH_2CO_2H$
21d	–$CH_2CH_2CH_2CH_2CO_2H$
21e	–CH=$CHCO_2H$
21f	–C(Me)=$CHCO_2H$
21g	–CH=C(Me)CO_2H
21h	–CH=CH–CH=$CHCO_2H$

Reagents and conditions: (a) NaOH aq., THF, EtOH, rt, overnight.

Chart 5

Results and Discussion

***In Vitro* Study** The synthesized compounds were examined for ACAT inhibitory activities in three ways (Table 2). We determined the enzymatic activity by using the human macrophage homogenates derived from the THP-1 cell line and the cellular activities by using both of the human macrophage and adrenal cell lines, THP-1 and H295R, respectively. The selectivity for macrophages over adrenal cells was evaluated by the ratio of each IC_{50} value (IC_{50} (adrenal)/IC_{50} (macrophage)).

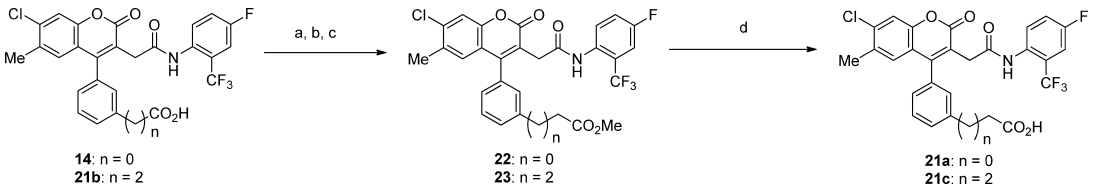
First, we replaced the chlorine at the 4-position on the anilide moiety of compound **2** with a fluorine in order to improve its activity on the basis of our previous structure–activities relationship study.[12] Compound **14** showed more potent inhibitory activity than compound **2** with respect to enzymatic activity, as we expected, and its selectivity for macrophage was slightly improved compared with compound **2**.

Thus, a further modification of the carboxylic acid moiety was examined with compound **14** in order to improve the potency and the selectivity for macrophages in cell-based assays. Compound **21a** showed a weaker enzymatic activity than compound **14**. Compound **21c** was the most potent ACAT inhibitor among the synthesized compounds.

Compounds **21b**—**h** showed similar potent inhibitory activities in macrophage cells despite possessing different enzymatic activities. On the other hand, the more lipophilic compounds, **21c** and **d**, showed more potent ACAT inhibitory activities in adrenal cells than the other compounds. These results show that the ACAT inhibitory activity might be more susceptible to lipophilicity in adrenal cells than in macrophage cells. The enhancement of adrenal ACAT inhibition led to a lower selectivity for macrophages. Compounds **21b** and **e** with log *D* values ranging from 2.8 to 3.1 showed potent inhibitory activities to ACAT in macrophages and high selectivity for macrophages over adrenal cells (26-, 28-fold, respectively). The selectivity was determined by a balance between the effects of the carboxyl acid moiety and the lipophilicity.

As a result of the *in vitro* studies, we chose compounds **21b** and **e** as candidates for further investigation in order to confirm the reduced adrenal toxicity properties. Compound **1** was also tested as a reference compound of potent adrenal toxicity.

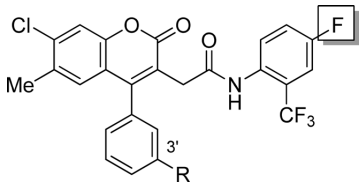
Adrenal Toxicological Test in Guinea Pig Adrenal toxicity was evaluated by a histopathologic examination in adrenal cortex 24 h after the intravenous administration of compounds **1**, **21b** and **e** in order to remove differences in their bioavailability (Table 3). Compound **1** showed adrenal toxic-



Reagents and conditions: (a) $(COCl)_2$, DMF (cat.), THF, rt, 0.5 h; (b) CH_2N_2 in Et_2O, rt, 2 h; (c) MeOH, Ag_2O, reflux, 2 h; (d) NaOH aq., THF, EtOH, rt, overnight.

Chart 6

Table 2. Effect of Introduction of Carboxylic Acid Moiety on ACAT Inhibitory Activity and Lipophylicity



Compds.	R	Enzyme assay[a] IC_{50} (nM)	Cell-based assay[b] IC_{50} (nM)			Log D[d]
			Macrophage	Adrenal	Selectivity[c]	
14	$-CO_2H$	57	62	584	9	2.65
21a	$-CH_2CO_2H$	>100	204	1940	7	2.73
21b	$-CH_2CH_2CO_2H$	40	9	249	28	3.06
21c	$-CH_2CH_2CH_2CO_2H$	7	7	63	10	3.30
21d	$-CH_2CH_2CH_2CH_2CO_2H$	28	10	39	4	3.66
21e	$-CH=CHCO_2H$	84	16	420	26	2.89
21f	$-C(Me)=CHCO_2H$	44	41	222	5	3.17
21g	$-CH=C(Me)CO_2H$	26	30	193	6	3.20
21h	$-CH=CH-CH=CHCO_2H$	81	29	256	9	3.22

a) Enzymatic ACAT inhibitory activity was determined by using homogenates from human THP-1 macrophages. *b*) Cellar ACAT inhibitory activity in human macrophage and adrenal was determined by using THP-1 and H295R cell lines, respectively. *c*) Selectivity was evaluated by the value of IC_{50} (adrenal)/IC_{50} (macrophage). *d*) Log D values were measured at pH 6.8.

Table 3. Adrenal Toxicity Test in Guinea Pigs[a]

Compds.	Adrenal toxicity test[a]		
	5 mg/kg, i.v.	15 mg/kg, i.v.	50 mg/kg, i.v.
1	+	NT	NT
21b	NT	—	+[b]
21e	NT	—	—

a) Compounds in dimethylsulfoxide were intravenously administered in guinea pig (n=3). The degree of adrenal toxicity was determined by histological diagnosis; +: evidence of adrenal cortical necrosis. —: no evidence of adrenal cortical necrosis. NT: not tested. *b*) Modest evidence of adrenal cortical necrosis was observed in one of three case.

Table 4. *Ex Vivo* Study in Atherosclerotic Rabbits[a]

Compds.	Aorta ACAT (% of control)	
	1 mg/kg, *p.o.*	10 mg/kg, *p.o.*
1	40±10*	8±1*
21e	54±4*	7±2*

a) Atherosclerotic male New Zealand white rabbits were used. Compounds or vehicle were administrated orally once daily for 3 d. The ACAT activity of thoracic aortas was determined, which was converted to percentage compared with mean ACAT activity of vehicle group. Data were represented as the mean±S.E.M. *$p<0.05$ *vs.* control (n=5—7).

ity at a dose of 5 mg/kg. This toxicity was characterized by adrenal cortical necrosis. Compound **21b** showed no evidence of toxicity at a dose of 15 mg/kg and moderate necrosis in only one of three cases at a dose of 50 mg/kg. Surprisingly, compound **21e** showed no adrenal toxicity even at a high dose of 50 mg/kg. Thus, compound **21e** may show high adrenal safety in sufficient plasma drug levels that are far in excess of the concentration necessary to inhibit ACAT in the walls of blood vessels.

***Ex Vivo* Study in Atherosclerotic Rabbit** In order to predict the anti-atherosclerotic effects in a short period, the ACAT activities in walls of blood vessels were measured after daily oral administration of compounds **1** and **21e** for 3 d (Table 4). Both compounds **1** and **21e** showed similar ACAT inhibitory activities at a dose of 1 and 10 mg/kg, respectively.

Conclusion

We investigated a (4-phenylcoumarin)acetanilide series in an effort to identify ACAT inhibitors with reduced adrenal toxicity. We hypothesized that the carboxylic acid moiety may lead to the identification of selective compounds and

evaluated the carboxylic acid analogs in cell-based assays with macrophages and adrenal cells. Adjustment of the lipophilicity of the compounds led to the discovery of (2*E*)-3-[7-chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acrylic acid (**21e**), which showed potent ACAT inhibitory activities in macrophages and selectivity of around 30-fold over adrenal cells. Compound **21e** also showed high adrenal safety in guinea pigs.

Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Varian Gemini 200 (200 MHz) or Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts are reported as δ values (ppm) downfield from internal tetramethylsilane of the indicated organic solution. Peak multiplicities are expressed as follow: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; dt doublet of triplet; br s, broad singlet; m, multiplet; br, broad. Coupling constants (*J* values) are given in hertz (Hz). Elemental analyses were carried out by Takeda Analytical Research Laboratories Ltd. Reaction progress was determined by thin layer chromatography (TLC) analysis on silica gel 60 F_{254} plate (Merck). Chromatographic purification was carried out on silica gel columns 60 (0.063—0.200 mm, Merck). Commercial reagents and solvents were used without additional purification. Abbreviations are used as follows: $CDCl_3$, deuterated chloroform; DMSO-d_6, dimethyl sulfoxide-d_6; EtOAc, ethyl acetate; DMF, *N*,*N*-dimethylformamide; MeOH, methanol; THF, tetrahydrofuran; EtOH, ethanol; CH_3CN, acetonitrile; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; AcOH, acetic acid.

(3-Bromophenyl)(4-chloro-2-hydroxy-5-methylphenyl)methanone (4) $AlCl_3$ (37.0 g, 277 mmol) was added portionwise to a stirred solution of 3-chloro-4-methylanisole (**3**) (37.6 g, 240 mmol) in chlorobenzene (100 ml) at 0 °C. Following the addition, 3-bromobenzoyl chloride (50.8 g, 231 mmol) was added dropwise, and the mixture was stirred at 100 °C for 30 min. After cooling to 0 °C, EtOAc (220 ml), MeOH (35 ml), and 4 N HCl aq. (150 ml) were successively added dropwise. The mixture was diluted with THF and extracted with EtOAc. The organic layer was separated, washed with 2 N HCl aq. and brine, dried over $MgSO_4$ and concentrated to give **4** (66.8 g, 89%) as pale yellow crystals; mp 115—117 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.28 (3H, s), 7.12 (1H, s), 7.36—7.42 (2H, m), 7.54—7.57 (1H, m), 7.72—7. 80 (2H, m), 11.70 (1H, s).

[4-(3-Bromophenyl)-7-chloro-6-methyl-2-oxo-2*H*-chromen-3-yl]acetic Acid (6) To a suspension of **4** (186 g, 571 mmol) in CH_3CN (400 ml) was added DBU (230 ml, 1530 mmol). A solution of ethyl succinyl chloride (157 g, 954 mmol) was added dropwise to the mixture at 40 °C for 1 h. After stirring for 0.5 h, water (450 ml) was added and the precipitate was collected by filteration and washed with EtOH to give crude ethyl [4-(3-bromophenyl)-7-chloro-6-methyl-2-oxo-2*H*-chromen-3-yl]acetate (**5**) (182 g). To the crude **5**, AcOH (1600 ml) and conc. HCl (600 ml) were added and the mixture was refluxed for 1 h. After concentration of the solvent, the residue was washed with water and dried. The obtained solid was crystallized from EtOAc to give **6** (166 g, 71%) as colorless crystals: mp >270 °C (decomp.); ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s), 3.33 (2H, s), 6.81 (1H, s), 7.25 (1H, d, *J*=7.6 Hz), 7.39 (1H, s), 7.45 (2H, m), 7.67 (1H, d, *J*=8.0 Hz); *Anal.* Calcd for $C_{18}H_{12}BrClO_4$: C, 53.03; H, 2.97. Found: C, 52.96; H, 3.00.

2-[4-(3-Bromophenyl)-7-chloro-6-methyl-2-oxo-2*H*-chromen-3-yl]-*N*-[4-fluoro-2-(trifluoro-methyl)phenyl]acetamide (12) To a solution of **6** (42.0 g, 103 mmol) in THF (400 ml) and DMF (5 drops) was added oxalyl chloride (11.0 ml, 124 mmol). The mixture was stirred at room temperature for 0.5 h. After concentration of the solvent, the residue was dissolved with THF (400 ml). To the solution was added 4-fluoro-2-(trifluoromethyl)aniline (**11a**) (14.7 ml, 120 mmol) and the mixture was stirred at room temperature overnight. The mixture was diluted with water and extracted with EtOAc. The organic layer was separated, washed with 1 N HCl aq., sat. $NaHCO_3$ aq and water successively, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was crystallized from EtOAc-THF to give **12** (60.0 g, 77%) as colorless crystals: mp 222—223 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.32 (3H, s), 3.38 (1H, d, *J*=14.0 Hz), 3.52 (1H, d, *J*=14.0 Hz), 6.86 (1H, s), 7.29 (3H, m), 7.45 (3H, m), 7.69 (1H, m), 7.98 (1H, m), 8.13 (1H, br s); *Anal.* Calcd for $C_{25}H_{15}BrClF_4NO_3$: C, 52.80; H, 2.66; N, 2.46. Found: C, 52.87; H, 2.80; N, 2.29.

2-Bromo-5-chloro-4-methylphenol (8) To a solution of **3** (98 g, 62.6 mmol) in AcOH (350 ml) was added dropwise bromine (100 g, 62.6 mmol) dissolved in AcOH (150 ml). After the addition, the mixture was stirred for 1 h. The resulting mixture was treated with $Na_2S_2O_3$ aq., followed by stirring for 1 h. After evaporation of the solvent, the obtained residue was diluted with water and extracted with EtOAc. The organic layer was washed with sat. $NaHCO_3$ aq. and water, dried over $MgSO_4$, and concentrated. The resulting oil was distilled under reduced pressure to give 1-bromo-4-chloro-2-methoxy-5-methylbenzene (130 g, 88%) as an oil. BBr_3 (52.5 ml, 555 mmol) was added dropwise to a stirred solution of obtained 1-bromo-4-chloro-2-methoxy-5-methylbenzene (63.3 g, 269 mmol) in CH_2Cl_2 (400 ml) at −78 °C under N_2. The resulting mixture was allowed to warm to room temperature and stirred for 2 h. The mixture was poured into ice-cold water and the organic layer was washed with brine, dried over $MgSO_4$ and concentrated to give **8** (59.4 g, 99%) as a pale yellow solid: ^{1}H-NMR ($CDCl_3$) δ: 2.28 (3H, s), 5.38 (1H, s), 7.04 (1H, s), 7.31 (1H, s).

(4-Chloro-2-hydroxy-5-methylphenyl)[3-(1,3-dioxolan-2-yl)phenyl] methanone (9) *n*-Buthyl lithium (1.6 M in hexane, 300 ml, 480 mmol) was added dropwise to a solution of **8** (48.7 g, 220 mmol) in THF (400 ml) at −78 °C. After 0.5 h, a solution of 3-(1,3-dioxolan-2-yl)benzaldehyde (39.2 g, 220 mmol) in THF (100 ml) was added dropwise and the resulting mixture was stirred for 1 h, then allowed to warm to room temperature. The mixture was quenched with sat. NH_4Cl aq. and extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$ and concentrated to give the oily product, which was used for the next reaction without further purification. A mixture of this product and MnO_2 (52.2 g, 600 mmol) in toluene (600 ml) was stirred at reflux with a Dean–Stark apparatus to remove water azeotropically. After 4 h, the mixture was filtered through a Celite pad and the filtrate was concentrated. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **9** (28.3 g, 44%) as an oil: ^{1}H-NMR ($CDCl_3$) δ: 2.26 (3H, s), 4.04—4.17 (4H, m), 5.88 (1H, s), 7.10 (1H, s), 7.39 (1H, s), 7.53 (1H, t, *J*=7.8 Hz), 7.63 (1H, dt, *J*=7.8, 1.5 Hz), 7.72 (1H, dt, *J*=7.8, 1.5 Hz), 7.77 (1H, t, *J*=1.5 Hz).

Ethyl {7-Chloro-4-[3-(1,3-dioxolan-2-yl)phenyl]-6-methyl-2-oxo-2*H*-chromen-3-yl}acetate (10) Ethyl succinyl chloride (12.7 ml, 88.8 mmol) was added dropwise to a solution of **9** (28.3 g, 88.8 mmol) and Et_3N (12.4 ml, 88.8 mmol) in THF (200 ml) at 0 °C, and the mixture was stirred for 0.5 h. After evaporation of the solvent, the residue was partitioned between water and EtOAc. The organic layer was washed with brine dried over $MgSO_4$ and concentrated. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give a yellow oil (27.4 g, 69%). The obtained product was dissolved in toluene (300 ml), and DBU (4.49 ml, 30.0 mmol) was added. The resulting mixture was stirred under reflux with a Dean–Stark apparatus to remove water azeotropically. After 2 h, the mixture was partitioned between water and EtOAc. The organic layer was washed with brine dried over $MgSO_4$ and concentrated. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **10** (21.3 g, 81%) as a pale yellow oil: ^{1}H-NMR ($CDCl_3$) δ: 1.22 (3H, t, *J*=7.0 Hz), 2.27 (3H, s), 3.34 (2H, s), 4.03—4.17 (6H, m), 5.83 (1H, s), 6.82 (1H, s), 7.25—7.29 (1H, m), 7.38—7.40 (2H, m), 7.55 (1H, t, *J*=7.8 Hz), 7.63 (1H, dt, *J*=7.8, 1.2 Hz).

2-[7-Chloro-4-(3-formylphenyl)-6-methyl-2-oxo-2*H*-chromen-3-yl]-*N*-[4-fluoro-2-(trifluoro-methyl)phenyl]acetamide (13a) A solution of **10** (2.80 g, 8.86 mmol) in AcOH (150 ml) and conc. HCl (75 ml) was refluxed for 1 h. After concentration of the solvent, the residue was washed with water and dried to give crude [7-chloro-4-(3-formylphenyl)-6-methyl-2-oxo-2*H*-chromen-3-yl]acetic acid (**7**) (2.50 g) as a solid, which was used for the next reaction without further purification. The crude **7** was converted into **13a** in a manner similar to that described for **12**.

Colorless crystals: mp 214—215 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.29 (3H, s), 3.36 (1H, d, *J*=14.0 Hz), 3.50 (1H, d, *J*=14.0 Hz), 6,80 (1H, s), 7.31 (2H, m), 7.48 (1H, s), 7.68 (1H, m), 7.77 (1H, t, *J*=7.7 Hz), 7.87 (1H, s), 7.98 (1H, m), 8.09 (1H, d, *J*=7.6 Hz), 8.19 (1H, br s), 10.11 (1H, s); *Anal.* Calcd for $C_{26}H_{16}ClF_4NO_4\cdot H_2O$: C, 59.68; H, 3.20; N, 2.68. Found: C, 59.45; H, 3.01; N, 2.63.

2-[7-Chloro-4-(3-formylphenyl)-6-methyl-2-oxo-2*H*-chromen-3-yl]-*N*-[4-chloro-2-(trifluoro-methyl)phenyl]acetamide (13b) The compound **13b** was prepared in a manner similar to that described for **13a**.

Colorless crystals: mp 232—233 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.29 (3H, s), 3.37 (1H, d, *J*=14.0 Hz), 3.51 (1H, d, *J*=14.0 Hz), 6,79 (1H, s), 7.47 (2H, m), 7.58 (1H, s), 7.66 (1H, d, *J*=7.4 Hz), 7.78 (1H, t, *J*=7.4 Hz), 7.87 (1H, s), 8.07 (2H, m), 8.28 (1H, br s), 10.11 (1H, s); *Anal.* Calcd for $C_{26}H_{16}Cl_2F_3NO_4\cdot H_2O$: C, 58.45; H, 3.20; N, 2.62. Found: C, 58.41; H, 3.03; N, 2.39.

3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-

6-methyl-2-oxo-2*H*-chromen-4-yl]benzoic Acid (14) Sodium chlorite (0.48 g, 5.31 mmol) was added to a solution of **13a** (0.78 g, 1.51 mmol), 2-methyl-2-butene (0.72 ml, 6.80 mmol) and sodium phosphate monobasic (0.18 g, 1.50 mmol) in *tert*-butanol (15 ml), THF (5 ml) and water. The mixture was stirred at room temperature for 2 h. The mixture was quenched with 1 N HCl aq., extracted with EtOAc. The organic layer was separated, washed with water, dried over $MgSO_4$ and concentrated *in vacuo* to give **14** (0.69 g, 86%) as colorless solid: mp 278—280 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.28 (3H, s), 3.35 (1H, d, J=16.0 Hz), 3.59 (1H, d, J=16.0 Hz), 6.82 (1H, s), 7.29 (2H, m), 7.45 (1H, d, J=9.2 Hz), 7.55 (2H, m), 7.75 (1H, m), 7.98 (1H, s), 8.22 (1H, d, J=7.6 Hz), 8.77 (1H, br s); *Anal.* Calcd for $C_{26}H_{16}ClF_4NO_5$: C, 58.49; H, 3.02; N, 2.62. Found: C, 58.41; H, 3.25; N, 2.43.

3-[7-Chloro-3-[2-[[4-chloro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]benzoic Acid (2) The compound **2** was prepared in a manner similar to that described for **14**.

Colorless crystals (92%): mp 270—272 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.28 (3H, s), 3.34 (1H, d, J=14.8 Hz), 3.57 (1H, d, J=14.8 Hz), 6.82 (1H, s), 7.40—7.70 (5H, m), 7.99 (2H, m), 8.23 (1H, d, J=7.8 Hz), 8.38 (1H, br s); *Anal.* Calcd for $C_{26}H_{16}Cl_2F_3NO_5$: C, 56.75; H, 2.93; N, 2.55. Found: C, 56.46; H, 3.02; N, 2.58.

Ethyl (2*E*)-3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acrylate (15) Ethyl acrylate (5.8 ml, 53.5 mmol), triethylamine (7.9 ml, 56.7 mmol), palladium acetate(II) (0.60 g, 2.67 mmol) and triphenylphosphine (1.30 g, 5.00 mmol) were added to a solution of **6** (30.0 g, 52.8 mmol) in DMF (300 ml) under N_2 atmosphere. The mixture was stirred at 100 °C for 5 h. The mixture was quenched with water, extracted with EtOAc. The organic layer was separated, washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane–EtOAc) and the product was crystallized from EtOAc to give **15** (16.7 g, 55%) as colorless crystals: mp 193—196 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.30 (3H, t, J=7.2 Hz), 2.30 (3H, m), 3.45 (1H, s), 4.26 (2H, q, J=7.2 Hz), 6.51 (1H, d, J=16.0 Hz), 6.86 (1H, s), 7.20—7.40 (3H, m), 7.48 (2H, m), 7.60 (1H, m), 7.73 (2H, m), 8.00 (1H, m), 8.18 (1H, br s); *Anal.* Calcd for $C_{30}H_{22}ClF_4NO_5$: C, 61.28; H, 3.77; N, 2.38. Found: C, 61.35; H, 3.89; N, 2.33.

Ethyl 3-[3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]propionate (16) To a solution of **15** (1.00 g, 1.70 mmol) in THF (50 ml) and EtOH (50 ml) was added Raney Ni (*ca.* 1.0 g). The mixture was stirred at room temperature under H_2 atmosphere overnight. The mixture was filtered through a Celite pad and the filterate was concentrated *in vacuo*. The residue was crystallized from EtOAc to give **16** (0.70 g, 69%) as colorless crystals: mp 121—123 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.21 (3H, t, J=7.0 Hz), 2.30 (3H, s), 2.67 (2H, t, J=7.5 Hz), 3.03 (2H, t, J=7.5 Hz), 3.45 (2H, s), 4.11 (2H, q, J=7.0 Hz), 6.91 (1H, s), 7.20—7.50 (7H, m), 7.99 (1H, m), 8.18 (1H, br s); *Anal.* Calcd for $C_{30}H_{24}ClF_3NO_5$: C, 61.08; H, 4.10; N, 2.37. Found: C, 61.03; H, 4.16; N, 2.41.

Methyl (2*E*)-3-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)-phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2-butenoate (17) The compound **17** was prepared in a manner similar to that described for **15**.

Colorless crystals (27%): mp 164—165 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s), 2.60 (3H, d, J=1.2 Hz), 3.46 (2H, s), 3.75 (3H, s), 6.23 (1H, d, J=1.2 Hz), 6.88 (1H, s), 7.20—7.27 (1H, m), 7.30—7.37 (2H, m), 7.45—7.46 (2H, m), 7.59 (1H, t, J=7.8 Hz), 7.64—7.68 (1H, m), 8.01 (1H, d, J=1.2 Hz), 8.22 (1H, s); *Anal.* Calcd for $C_{30}H_{22}ClF_4NO_5$: C, 61.28; H, 3.77; N, 2.38. Found: C, 61.36; H, 3.85; N, 2.33.

Ethyl (2*E*)-3-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)-phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2-methylacrylate (20) NaH (65.5 mg, 1.80 mmol) was added portionwise to a solution of triethyl 2-phosphonopropionate (0.536 g, 2.25 mmol) in THF (10 ml) at 0 °C and the mixture was stirred for 30 min. **13a** (0.777 g, 1.50 mmol) was added and the resulting mixture was stirred at the same temperature for 30 min and at room temperature for 2 h. The mixture was diluted with water and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel column chromatography (hexane–EtOAc–$CHCl_3$) to give **20** (346 mg, 61%) as colorless crystals (0.84 g, 93%): mp 172—174 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, t, J=7.2 Hz), 2.11 (3H, d, J=1.5 Hz), 2.29 (3H, s), 3.42 (1H, d, J=13.8 Hz), 3.52 (1H, d, J=13.8 Hz), 4.27 (2H, q, J=7.2 Hz), 6.87 (1H, s), 7.19—7.26 (1H, m), 7.29—7.32 (3H, m), 7.45 (1H, s), 7.55—7.62 (2H, m), 7.70 (1H, d, J=1.5 Hz), 7.97 (1H, dd, J=9.0, 5.2 Hz), 8.13 (1H, s); *Anal.* Calcd for $C_{31}H_{24}ClF_4NO_5$: C, 61.85; H, 4.02; N, 2.33. Found: C, 61.84; H, 4.22; N, 2.40.

Ethyl (2*E*,4*E*)-5-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)-phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2,4-pentadienoate (18) The compound **18** was prepared in a manner similar to that described for **20**.

Colorless crystals (29%): mp 199—201 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.32 (3H, t, J=7.2 Hz), 2.30 (3H, s), 3.46 (2H, s), 4.23 (2H, q, J=7.2 Hz), 5.98 (1H, d, J=15.4 Hz), 6.90—6.95 (3H, m), 7.19—7.66 (8H, m), 7.99 (1H, dd, J=9.2, 5.0 Hz), 8.17 (1H, s); *Anal.* Calcd for $C_{32}H_{24}ClF_4NO_5$: C, 62.60; H, 3.94; N, 2.28. Found: C, 62.71; H, 3.90; N, 2.26.

Ethyl 5-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)phenyl]-amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}pentanoate (19) The compound **19** was prepared in a manner similar to that described for **16**.

Colorless crystals (65%): mp 141—142 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.23 (3H, t, J=7.2 Hz), 1.65—1.70 (4H, m), 2.29—2.33 (5H, m), 2.69—2.74 (2H, m), 3.43 (1H, d, J=13.8 Hz), 3.50 (1H, d, J=13.8 Hz), 4.10 (2H, q, J=7.2 Hz), 6.91 (1H, s), 7.13—7.35 (5H, m), 7.43—7.48 (2H, m), 7.98 (1H, dd, J=8.7, 4.8 Hz), 8.16 (1H, s); *Anal.* Calcd for $C_{32}H_{28}ClF_4NO_5$: C, 62.19; H, 4.57; N, 2.27. Found: C, 62.10; H, 4.44; N, 2.22.

(2*E*)-3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acrylic Acid (21e) To a solution of **15** (575 mg, 0.98 mmol) in THF (10 ml) and EtOH (5 ml) was added 1 N NaOH aq. (5 ml). The mixture was stirred at room temperature overnight. The mixture was neutralized with 1 N HCl aq. and extracted with EtOAc. The organic layer was separated, washed with water, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was crystallized from EtOAc to give **21e** (346 mg, 61%) as colorless crystals: mp 260—262 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, m), 3.46 (2H, s), 6.52 (1H, d, J=15.8 Hz), 6.86 (1H, s), 7.20—7.40 (4H, m), 7.60—7.80 (3H, m), 7.82 (1H, d, J=15.8 Hz), 7.98 (1H, m), 8.23 (1H, br s); *Anal.* Calcd for $C_{28}H_{18}ClF_4NO_5$: C, 60.06; H, 3.24; N, 2.50. Found: C, 59.94; H, 3.19; N, 2.45.

Compounds **21b**, **d** and **f**—**h** were prepared in a manner similar to that described for **21e**.

3-[3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]propionic Acid (21b) Colorless crystals (91%): mp 212—214 °C; ^{1}H-NMR ($CDCl_3$) δ: 2.29 (3H, s), 2.68 (2H, t, J=7.2 Hz), 3.01 (2H, t, J=7.2 Hz), 3.37 (1H, d, J=13.5 Hz), 3.54 (1H, d, J=13.5 Hz), 6.89 (1H, s), 7.10—7.50 (7H, m), 7.90 (1H, m), 8.46 (1H, br s); *Anal.* Calcd for $C_{28}H_{20}ClF_4NO_5$: C, 59.88; H, 3.88; N, 2.52. Found: C, 59.85; H, 3.59; N, 2.49.

5-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}pentanoic Acid (21d) Colorless crystals (70%): mp 179—181 °C; ^{1}H-NMR ($CDCl_3$) δ: 1.60—1.76 (4H, m), 2.29 (3H, s), 2.35 (2H, t, J=6.6 Hz), 2.72 (2H, t, J=6.6 Hz), 3.47 (2H, s), 6.91 (1H, s), 7.13—7.36 (5H, m), 7.43—7.51 (2H, m), 7.97 (1H, dd, J=9.0, 5.0 Hz), 8.22 (1H, s); *Anal.* Calcd for $C_{30}H_{24}ClF_4NO_5$: C, 61.08; H, 4.10; N, 2.37. Found: C, 60.86; H, 4.05; N, 2.29.

(2*E*)-3-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2-butenoic Acid (21f) Colorless crystals (66%): mp 252—255 °C; ^{1}H-NMR (DMSO-d_6) δ: 2.27 (3H, s), 2.50 (3H, s), 3.34 (2H, s), 6.17 (1H, s), 6.93 (1H, s), 7.34—7.80 (8H, m), 9.67 (1H, s), 12.23 (1H, s); *Anal.* Calcd for $C_{29}H_{20}ClF_4NO_5$: C, 60.69; H, 3.51; N, 2.44. Found: C, 60.66; H, 3.56; N, 2.35.

(2*E*)-3-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2-methylacrylic Acid (21g) Colorless crystals (72%): mp 268—271 °C; ^{1}H-NMR (DMSO-d_6) δ: 2.00 (3H, d, J=1.2 Hz), 2.26 (3H, s), 3.27—3.43 (2H, m), 6.94 (1H, s), 7.30—7.41 (3H, m), 7.48—7.55 (1H, m), 7.60 (1H, dd, J=9.0, 2.7 Hz), 7.64—7.68 (3H, m), 7.70 (1H, s), 9.65 (1H, s); *Anal.* Calcd for $C_{29}H_{20}ClF_4NO_5$: C, 60.69; H, 3.51; N, 2.44. Found: C, 60.54; H, 3.68; N, 2.27.

(2*E*,4*E*)-5-{3-[7-Chloro-3-(2-{[4-fluoro-2-(trifluoromethyl)-phenyl]amino}-2-oxoethyl)-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl}-2,4-pentadienoic Acid (21h) Colorless crystals (87%): mp >300 °C; ^{1}H-NMR (DMSO-d_6) δ: 2.26 (3H, s), 3.41 (2H, s), 5.98 (1H, d, J=14.6 Hz), 6.92 (1H, s), 7.12—7.18 (2H, m), 7.24—7.41 (3H, m), 7.47—7.67 (4H, m), 7.71—7.81 (2H, m), 9.65 (1H, s), 12.30 (1H, s); *Anal.* Calcd for $C_{30}H_{20}ClF_4NO_5$: C, 61.50; H, 3.44; N, 2.39. Found: C, 61.61; H, 3.68; N, 2.26.

Methyl 3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acetate (22) To a solution of **14** (2.0 g, 3.75 mmol) in THF (100 ml) and DMF (3 drops) was added oxalyl chloride (0.40 ml, 4.59 mmol). The mixture was stirred at room temperature for 0.5 h. After concentration of the solvent, the residue was dis-

solved with THF (50 ml). This solution was added to a solution of diazomethane in diethylether (50 ml), prepared from *N*-methyl-*N'*-nitroso-*N*-nitroguanizine (3.0 g, 20.4 mmol), at room temperature. After stirring at room temperature for 2 h, the mixture was concentrated *in vacuo*. To the residue was added MeOH (100 ml), followed by silver oxide (1.2 g, 5.18 mmol). The mixture was refluxed for 2 h. The mixture was filtered through a Celite pad and the filterate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **22** as a pale yellow solid, which was used for the next reaction without further purification: ^{1}H-NMR ($CDCl_3$) δ: 2.30 (3H, s), 3.42 (1H, d, J=10.2 Hz), 3.47 (1H, d, J=10.2 Hz), 3.70 (3H, s), 3.72 (3H, s), 6.94 (1H, s), 7.25—7.33 (4H, m), 7.43—7.52 (4H, m), 7.97 (1H, m), 8.16 (1H, br s).

3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]acetic Acid (21a) The compound **21a** was prepared in a manner similar to that described for **21e**.

Colorless crystals (8% from **14**): mp 173—175 °C; ^{1}H-NMR (DMSO-d_6) δ: 2.25 (3H, s), 3.38 (2H, s), 3.62 (2H, s), 6.97 (1H, s), 7.25 (2H, m), 7.47—7.63 (5H, m), 7.70 (1H, s), 9.69 (1H, br s); *Anal.* Calcd for $C_{27}H_{18}ClF_4NO_5 \cdot H_2O$: C, 58.23; H, 3.44; N, 2.52. Found: C, 58.11; H, 3.61; N, 2.45.

Methyl 4-[3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]-amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]butanoate (23) The compound **23** was prepared in a manner similar to that described for **22**.

A pale yellow solid: ^{1}H-NMR ($CDCl_3$) δ: 2.00 (2H, m), 2.30 (3H, s), 2.36 (2H, t, J=7.6 Hz), 2.74 (2H, t, J=7.7 Hz), 3.46 (2H, s), 3.64 (3H, s), 6.92 (1H, s), 7.15—7.37 (5H, m), 7.48 (2H, m), 8.00 (1H, m), 8.19 (1H, br s); *Anal.* Calcd for $C_{30}H_{24}ClF_4NO_5$: C, 61.08; H, 4.10; N, 2.37. Found: C, 60.95; H, 3.98; N, 2.24.

4-[3-[7-Chloro-3-[2-[[4-fluoro-2-(trifluoromethyl)phenyl]amino]-2-oxoethyl]-6-methyl-2-oxo-2*H*-chromen-4-yl]phenyl]butanoic Acid (21c) The compound **21c** was prepared in a manner similar to that described for **21a**.

Colorless crystals (19% from **21b**): mp 195—196 °C; ^{1}H-NMR (DMSO-d_6) δ: 1.84 (2H, m), 2.24 (2H, m), 2.49 (3H, s), 2.68 (2H, m), 3.33 (2H, s), 6.92 (1H, s), 7.18 (2H, m), 7.39—7.64 (5H, m), 7.71 (1H, s), 9.65 (1H, br s), 12.06 (1H, br); *Anal.* Calcd for $C_{29}H_{22}ClF_4NO_5$: C, 60.48; H, 3.85; N, 2.43. Found: C, 60.16; H, 3.75; N, 2.28.

Inhibitory Effects of Compounds on ACAT Activity ACAT activities were determined by incorporation of [^{3}H] oleoyl-CoA into cholesteryl esters using homogenates from human THP-1 macrophages. Inhibitory effects of compounds on ACAT activity were determined by measuring the radioactivities of [^{3}H] cholesteryl oleate fraction with or without compounds and these IC_{50} values calculated from means of ACAT inhibition in duplicate measurements.

Measurements of CE Formation in Cultured Cells Monocytic THP-1 and adrenal H295R cells were used for measurements of cholesteryl ester formation in cells. Differentiated THP-1 macrophages and H295-R cells were cholesterol-loaded by exposure to rabbit β very low density lipoprotein (150 μg cholesterol/ml) for one day and then cellular cholesteryl ester formation was estimated by the incorporation of [^{3}H] oleic acid into cholesteryl ester. Inhibitory effects of compounds on ACAT activity were determined by measuring the radioactivities of intracellular [^{3}H] cholesteryl oleate fraction with or without compounds and these IC_{50} values calculated from means of ACAT inhibition in triplicate measurements.

Adrenal Toxicological Test in Guinea Pigs Compounds were dissolved in dimethysulfoxide and intravenously administered in guinea pig (n=3). After 24 h administration, animals were sacrificed and the adrenal glands were dissected. The degree of toxicity was determined by histological diagnosis.

***Ex Vivo* Study in Atherosclerotic Rabbits** Atherosclerotic male New Zealand white rabbits (14-week-old) were used. Animals were fed a chow diet supplemented with 0.5% cholesterol, 3% peanut oil, and 3% coconut oil to induce atherosclerotic lesions for 8 weeks followed by a chow diet for 6 weeks. Animals were orally given compounds or vehicle once daily for 3 d (**1**: solution in Gelucire,[12] **21e**: suspension in 0.5% methylcellulose). About 2 h after the last administration, animals were sacrificed under the anesthesia and thoracic aortas were dissected and frozen. The ACAT activity of these tissue homogenates were determined by incorporation of [^{3}H] oleoyl-CoA into cholesteryl esters. All homogenate samples were determined ACAT activity by incorporated [^{3}H] oleoyl-CoA per protein concentration. ACAT activity of each sample was converted to percentage compared with mean ACAT activity of vehicle group. Data were represented as the mean±S.E.M.

Acknowledgement We would like to thank Dr. K. Kato, Dr. Y. Imura for their encouragement and help advice. We would like to thank Dr. T. Watanabe for toxicological test. We would like to thank Mr. M. Nakamura for pharmacological directions.

References

1) Libby P., *J. Intern. Med.*, **263**, 517—527 (2008).
2) Lee O., Chang C. C., Lee W., Chang T. Y., *J. Lipid Res.*, **39**, 1722—1727 (1998).
3) Buhman K. F., Accad M., Farese R. V., *Biochim. Biophys. Acta*, **1529**, 142—154 (2000).
4) Rudel L. L., Lee R. G., Cockman T. L., *Curr. Opin. Lipidol.*, **12**, 121—127 (2001).
5) Chang T. Y., Li B. L., Chang C. C., Urano Y., *Am. J. Physiol. Endocrinol. Metab.*, **297**, E1—E9 (2009).
6) Chang T. Y., Chang C. C., Lin S., Yu C., Li B. L., Miyazaki A., *Curr. Opin. Lipidol.*, **12**, 289—296 (2001).
7) Oelkers P., Behari A., Cromley D., Billheimer J. T., Sturley S. L., *J. Biol. Chem.*, **273**, 26765—26771 (1998).
8) Miyazaki A., Kanome T., Watanabe T., *Curr. Drug Targets Cardiovasc. Haematol. Disord.*, **5**, 463—469 (2005).
9) Ikenoya M., Yoshinaka Y., Kobayashi H., Kawamine K., Shibuya K., Sato F., Sawanobori K., Watanabe T., Miyazaki A., *Atherosclerosis*, **191**, 290—297 (2007).
10) Bocan T. M., Krause B. R., Rosebury W. S., Mueller S. B., Lu X., Dagle C., Major T., Lathia C., Lee H., *Arterioscler. Thromb. Vasc. Biol.*, **20**, 70—79 (2000).
11) Delsing D. J., Offerman E. H., van Duyvenvoorde W., van Der Boom H., de Wit E. C., Gijbels M. J., van Der Laarse A., Jukema J. W., Havekes L. M., Princen H. M., *Circulation*, **103**, 1778—1786 (2001).
12) Ogino M., Fukui S., Nakada Y., Tokunoh R., Itokawa S., Kakoi Y., Nishimura S., Sanada T., Fuse H., Kubo K., Wada T., Marui S., *Chem. Pharm. Bull.*, **59**, 1268—1273 (2011).
13) Junquero D., Pilon A., Carilla-Durand E., Patoiseau J. F., Tarayre J. P., Torpier G., Staels B., Fruchart J. C., Colpaert F. C., Clavey V., Delhon A., *Biochem. Pharmacol.*, **61**, 387—398 (2001).
14) Sliskovic D. R., Picard J. A., O'Brien P. M., Liao P., Roark W. H., Roth B. D., Anderson M. A., Mueller S. B., Bocan T. M., Bousley R. F., Hamelehle K. L., Homan R., Reindel J. F., Stanfield R. L., Turluck D., Krause B. R., *J. Med. Chem.*, **41**, 682—690 (1998).
15) Robertson D. G., Breider M. A., Milad M. A., *Toxicol. Sci.*, **59**, 324—334 (2001).
16) Takahashi K., Kasai M., Ohta M., Shoji Y., Kunishiro K., Kanda M., Kurahashi K., Shirahase H., *J. Med. Chem.*, **51**, 4823—4833 (2008).
17) Chatterjea J. N., Jha H. C., Chattopadhyaya A. K., *Justus Liebigs Ann. Chem.*, 1126—1131 (1974).