Synthesis of 3-Substituted Isocoumarins and Their Inhibitory Effects on Degranulation of RBL-2H3 Cells Induced by Antigen

Ai KURUME,[a] Yasuhiro KAMATA,[a] Masayuki YAMASHITA,*,[a] Qilong WANG,[b] Hisashi MATSUDA,[b] Masayuki YOSHIKAWA,[b] Ikuo KAWASAKI,[a,c] and Shunsaku OHTA[a]

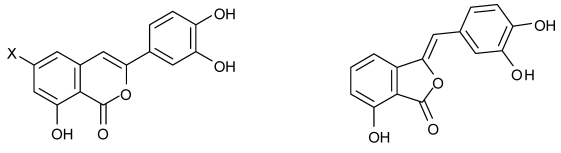
[a] *Department of Functional Molecular Chemistry, Kyoto Pharmaceutical University;* [b] *Department of Pharmacognosy, 21st Century COE Program, Kyoto Pharmaceutical University; Misasagi, Yamashina-ku, Kyoto 607–8414, Japan: and* [c] *School of Pharmaceutical Sciences, Mukogawa Women's University; 11–68 Koshien Kyuban-cho, Nishinomiya 663–8179, Japan.*
Received March 3, 2008; accepted July 4, 2008; published online July 9, 2008

Eleven 3-substituted isocoumarins and a benzylidenephthalide were synthesized through thermal cyclization reaction of δ- and γ-ketoamides, respectively. Subsequent deprotection of the hydroxyl groups of the resulting isocoumarin and benzylidenephthalide compounds afforded thunberginols A, B, and F, respectively, which originated from the processed leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO. The synthesized isocoumarins and thunberginols were evaluated for their anti-allergic activity, in which thunberginol B exhibited the highest inhibitory potency on the degranulation of RBL-2H3 cells induced by antigen. Structure–activity relationship studies were carried out to determine the necessary substituents on the 3-phenylisocoumarin skeleton for inhibitory activity.

Key words thunberginol; antiallergy; isocoumarin

Although excellent anti-allergic agents have been developed, recent increases in the number of patients in Japan with allergies such as pollinosis and asthma necessitate more effective and safe anti-allergic agents. In a previous study, anti-allergic thunberginols A (**1a**), B (**1b**), and F (**1c**), which were isolated from Hydrangeae Dulcis Folium, the processed leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO,[1—6] were shown to substantially inhibit the degranulation of rat peritoneal mast cells and rat basophilic leukemia (RBL-2H3) cells stimulated by calcium ionophore A23187 or antigen. Of the three, **1b** was reported to be a potential anti-allergic compound.[5,6] Because **1a**, **1b**, and **1c** can be isolated from the herbs in only extremely low yields (**1a**: 0.0086%, **1b**: 0.0016%, **1c**: 0.0028%), investigations of the compounds' anti-allergic effects, metabolism, and safety using animal models require an efficient synthetic methodology. In a previous study, we reported the synthesis of 3-substituted isocoumarin derivatives and its application towards the total synthesis of thunberginol A.[7] Herein, we report the preparation of 3-substituted isocoumarins and an improved synthetic chart of thunberginols. Moreover, the resulting compounds are evaluated for their inhibitory effects on degranulations induced by an antigen in RBL-2H3 cells.

Chemistry Designed as precursors for isocoumarins **4**, δ-ketoamides **3** were prepared *via* benzylic metalation involving the directing effects of the amide group.[8] Lithiation conditions for **2a** were investigated using various lithiating agents such as lithium diisopropylamide (LDA), *sec*- and *tert*-BuLi, followed by treatment of *N*,*N*-dimethylbenzamide as the electrophile. The maximum yield of δ-ketoamide **3a** was obtained when *N*,*N*-diethyl-2-toluamide was lithiated with *tert*-BuLi at −65 °C. The generality of these reactions under optimized conditions was proved as shown in Table 1.



1a (X = H, thunberginol A)
1b (X = OH, thunberginol B)

1c (thunberginol F)

Fig. 1. Chemical Structure of Thunberginols A, B and F

Among the various cyclization conditions for the conversion of ketoamide **3a** to 3-phenylisocoumarin **4a**, as summarized in Table 2, the maximum yield (91%) was obtained under only refluxing AcOH for 3 h (entry 3).[7] Under the same reaction conditions, *N*,*N*-diethyl-δ-ketoamides **3b**—**j** were readily cyclized to the corresponding 3-substituted isocoumarins **4b**—**j** in good to excellent yields (99—76%, Table 1), which can be attributed to the thermodynamic stability of the resulting aromatic isocoumarin nucleus.

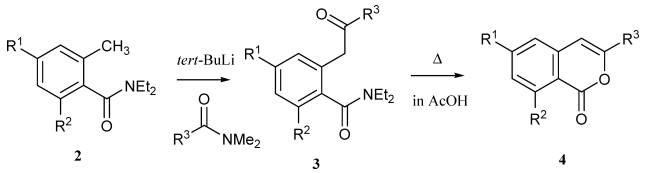
As shown in Table 3, demethylation of **4c**, **4h** and **4i** was performed *via* treatment with BBr_3. Deprotection of **4c** and **4h** proceeded smoothly under relatively mild conditions (6 eq of BBr_3, −78 °C to rt) to give **1d** (entry 1, 94% yield) and thunberginol A (**1a**, entry 2, 92% yield), respectively. In contrast, treatment of **4i** with BBr_3 under similar conditions gave the partially deprotected 6-methylated thunberginol B (**1e**, entry 3, 97% yield). Despite the report of complete demethylation of **4i** using BBr_3 (10 eq) at rt by Rossi *et al.*,[9] in our hands, **1b** was obtained in low yields, along with some decomposition compounds, only after treatment of **4i** with excess BBr_3 (10 eq) in refluxing 1,1,2,2-tetrachloroethane.

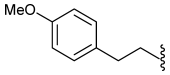
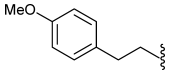
To improve the yield of **1b**, our synthetic methodology was modified using 4-MOM-protected δ-ketoamide **3k** (Table 1, entry 11) as the precursor (Chart 1). The two-step isocoumarin synthesis, as described above, was applied to amide **2d** (Table 1, entry 11) to afford 6-deprotected isocoumarin **4k** (79% overall yield from **2d**). Deprotection of **4k** using BBr_3 (6 eq) at −78 °C to rt proceeded as expected to give **1b** in 84% yield (Table 3, entry 4).

Subsequently, the synthesis of **1c**, a (*Z*)-3-benzylidenephthalide that was co-isolated with thunberginols A and B from the same plant,[1] was carried out using the above reaction chart. As shown in Chart 2, *N*,*N*-diethyl-2-methoxybenz amide **9** was acylated *via* the *ortho*-lithiation process[8] using

* To whom correspondence should be addressed. e-mail: yamasita@mb.kyoto-phu.ac.jp

Table 1. Synthesis of δ-Ketoamide **3** and Isocoumarin **4**



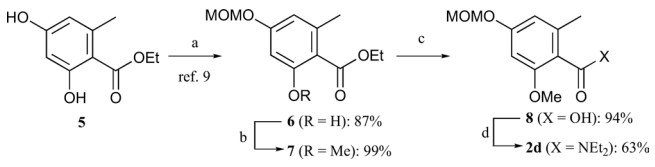
Entry	**2**			*N*,*N*-Dimethylamide	δ-Ketoamide **3**; yield (%)	Isocoumarin **4**; yield (%)
		R^1	R^2	R^3		
1	**2a**:	H	H	Ph-	**3a**; 60	**4a**; 91
2	**2a**:	H	H	4-MeO–C_6H_4–	**3b**; 82	**4b**; 91
3	**2a**:	H	H	3,4-diMeO–C_6H_3–	**3c**; 82	**4c**; 98
4	**2a**:	H	H	Me	**3d**; 81	**4d**; 87
5	**2a**:	H	H	*n*-Hexyl	**3e**; 73	**4e**; 99
6	**2a**:	H	H	Cyclohexyl	**3f**; 66	**4f**; 76
7	**2a**:	H	H		**3g**; 82	**4g**; 96
8	**2b**:	H	MeO	3,4-diMeO–C_6H_3–	**3h**; 73	**4h**; 81
9	**2c**:	MeO	MeO	3,4-diMeO–C_6H_3–	**3i**; 68	**4i**; 99
10	**2c**:	MeO	MeO		**3j**; 93	**4j**; 91
11	**2d**:	MOMO	MeO	3,4-diMeO–C_6H_3–	**3k**; 85	**4k**[a]; 93

a) **4k**: R^1=OH; R^2=MeO; R^3=3,4-diMeO–C_6H_3–.

Table 2. Cyclization of δ-Ketoamide **3a** to Isocoumarin **4a**

Entry	Additive	Solvent	Temp. (°C)	Time (h)	Yield of **4a** (%)
1	—	AcOH	50	6	22
2	—	AcOH	100	3	88
3	—	AcOH	Reflux	3	91
4	HCl[a]	THF[a]	50	6	13
5	*t*-BuOLi[b]	THF	Rt	12	0[c]
6	—	Toluene	100	3	25
7	—	Xylene	Reflux	2	86

a) 10% HCl (aq.)/THF=1 : 1. *b*) 1 eq of *t*-BuOLi was used. *c*) Recovery of **3a**.

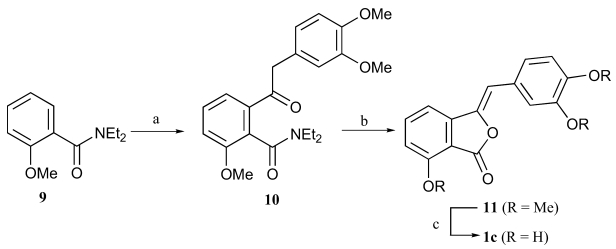


Reagents and conditions: (a) K_2CO_3, MOMCl, 87%; (b) NaH, MeI, 99%; (c) KOH, MeOH, 94%; (d) EDAC, DMAP, Et_2NH, 63%.

Chart 1

2-(3,4-dimethoxyphenyl)-*N*,*N*-dimethylacetamide to give γ-ketoamide **10** in 11% yield. This low yield can be attributed to anion migration of lithiated **9** to the benzylic position of the *N*,*N*-dimethylacetamide. Next, γ-ketoamide **10** was readily converted to (*Z*)-3-benzylidenephthalide **11** (81% yield), then smoothly demethylated to give **1c** in 98% yield.

Biological Study Along with the release of histamine, degranulation of granules in mast cells or basophils also causes the release of an enzyme, β-hexosaminidase. Accordingly, determination of β-hexosaminidase activity, as a marker of mast cell or basophil degranulation, offers an alternative method of evaluating anti-allergic compounds using passive cutaneous anaphylaxis (PCA) reactions in laboratory animals.[5,11,12] In the present study, synthetic isocoumarins **1d**, **1e**, **4a**—**c**, **4f**—**j**, and 3-benzylidenephthalide **11** evaluated for their effects on the release of β-hexosaminidase from RBL-2H3 cells in order to investigate the relationship between substituent groups on the 3-phenylisocoumarin skeleton and biological activities. The results were compared to those of naturally isolated **1a** (IC_{50}=17 μM), **1b** (5.7 μM), and **1c** (19 μM),[5] of which, **1b** possessed the highest potency. Among the synthetic compounds, **1e** exhibited substantial inhibition of degranulation, which was nearly equivalent to those of **1a** and **1c** (Table 4). Furthermore, to confirm that the inhibitory effects of the active compounds **4a**—**c**, **4h**, and **1a**—**e** are due to the inhibition of degranulation, but not the false positive due to the inhibition of enzyme activity of β-hexosaminidase, effects of the compounds on the enzyme activity were examined. As a result, each compound showed only a weak inhibition against enzyme activity of β-hexosaminidase less than 10% at 100 μM (data not shown).



Reagents and conditions: (a) *tert*-BuLi, 2-(3,4-dimethoxyphenyl)-*N*,*N*-dimethylacetamide, TMEDA, THF, −78 °C to rt, 11%; (b) AcOH, reflux, 81%; (c) BBr_3, DCM, −78 °C to rt, 98%.

Chart 2

Table 3. Demethylation of **4**[a)]

4c, 4h, 4i, 4k **1a, 1b, 1d, 1e**

Entery	**4**			**1**			
		X	Y		X′	Y′	Yield of **1** (%)
1	**4c**:	H	H	**1d**:	H	H	94
2	**4h**:	H	MeO	**1a** (thunberginol A):	H	OH	92
3	**4i**:	MeO	MeO	**1e**:	MeO	OH	97
4	**4k**:	OH	MeO	**1b** (thunberginol B):	OH	OH	84

a) All reaction was carried out with an excess BBr_3 in CH_2Cl_2 at −78 °C to rt.

Table 4. Effects of Synthetic Compounds on DNP-BSA-Induced Degranulations in RBL-2H3 Cells Sensitized with Anti-DNP IgE

Compounds	Conc. (μM)	Inhibition (%)	IC_{50} (μM)	Compounds	Conc. (μM)	Inhibition (%)	IC_{50} (μM)
4a	0	0.0±2.9		**4h**	0	0.0±2.9	
	30	−1.4±3.4	100		10	26.6±3.6**	100
	60	25.3±2.9**			30	36.4±3.0**	
	100	53.3±3.1**			100	49.4±1.4**	
4b	0	0.0±4.5		**4i**	0	0.0±4.3	
	3	12.6±6.3			10	26.3±2.7**	>100
	10	22.4±4.2**	100		30	36.6±5.4**	
	30	40.9±4.7**			100	44.1±2.3**	
	100	50.3±2.9**					
				4j	0	0.0±4.2	
4c	0	0.0±4.5			100	8.1±3.9	>100
	3	3.8±5.0					
	10	33.5±3.5**	20	**1d**	0	0.0±1.6	
	30	69.5±2.4**			10	5.44±2.1	
	100	79.6±1.3**			30	15.2±2.2**	84
					100	63.3±2.1**	
4f	0	0.0±4.2					
	100	−10.3±5.6	>100	**1e**	0	0.0±4.4	
					10	28.6±5.7**	18
4g	0	0.0±4.2			30	87.6±2.6**	
	100	26.6±4.3**	>100		100	104.7±0.8**	
				11	0	0.0±2.3	>100
					100	30.8±1.5**	

Each value represents the mean±S.E.M. (n=4). Significantly different from control, ** $p<0.01$.

A previous study based on structure–activity relationships (SAR)[3,5)] has demonstrated the necessity of the hydroxyl groups at the 3′-, 4′-, 6-, and 8-positions for activity. Activities of our synthetic isocoumarins showed a similar trend [**4a** (100 μM)<**1d** (84 μM)<**1a** (17 μM)<**1b** (5.7 μM)]. In addition, permethylation of the hydroxyl groups in **1a** and **1b** as well as **1c** significantly reduced their activities [**4h** (100 μM)<**1a** (17 μM), **4i** (>100 μM)<**1b** (5.7 μM), **11** (>100 μM)<**1c** (19 μM)], with the exception of the stronger activity of **4c** than that of **1d** [**4c** (20 μM)>**1d** (84 μM)]. Although methylation of the 6-hydroxyl group in **1b** reduced its activity, the activity of **1e** remained equipotent to that of **1a** [**1e** (18 μM)=**1a** (17 μM)<**1b** (5.7 μM)].

To date, detailed mechanisms of **1a** and **1b**, including those of the target molecules, have yet to be clarified. Recently, photolabile ligands have been employed in drug discovery.[13)] Because an isocoumarin structure is fluorescent, and because substitution at the 6-position does not markedly reduce activity, synthetic **1a** and **1b** can be utilized as the substrates for the development of photolabile ligands to investigate target molecules.

Experimental

General Melting point was measured using a Yanaco MP micro-melting-point apparatus and are uncorrected. IR spectra were taken using a Shimadzu IR-435 spectrophotometer. NMR spectra were measured using JEOL-EX 270 (^{1}H: 270 MHz), Varian XL-300 (^{1}H: 300 MHz; ^{13}C: 75 MHz), or Varian UNITY INOVA 400NB (^{1}H: 400 MHz; ^{13}C: 100 MHz); the chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane as the internal standard. MS and HR-MS (EI) were measured using a Hitachi M-80 or JEOL JMS BU-20 spectrometer. Purifications using column chromatography was carried out on silica gel (Merck Art. 7737).

General Procedure for the Synthesis of δ-Ketoamides (3) and the γ-Ketoamide 10, Synthesis of *N*,*N*-Diethyl-2-(2-oxo-2-phenylethyl)benzamide (3a) as an Example *tert*-BuLi (1.9 M in pentane; 1.1 ml, 2.1 mmol) was added to a stirred solution of **2a** (383 mg, 2 mmol) in THF (4 ml) under N_2 at −65 °C. After stirring for 1 h at the same temperature, a solution of *N*,*N*-dimethylbenzamide (298 mg, 2 mmol) in THF (2 ml) was added to the reaction mixture and the whole was stirred for 4 h at ambient temperature.

H_2O (5 ml) was added to the mixture, and after evaporation of the solvent the products were extracted with AcOEt (20 ml×2). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give an oily residue, which was purified by column chromatography (AcOEt/*n*-hexane=1/2) to give **3a** as a viscous oil (356 mg, 60%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.02 and 1.07 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 3.08—3.62 (4H, br, 2×$NC\underline{H}_2CH_3$), 4.41 (2H, br s, $COCH_2$), 7.23—7.59 (7H, m, Ar), 8.00—8.03 (2H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1683, 1615. HR-MS *m/z*: 295.1547 (Calcd for $C_{19}H_{21}NO_2$: 295.1570).

***N*,*N*-Diethyl-2-[2-(4-methoxyphenyl)-2-oxoethyl]benzamide (3b)** Starting with **2a** and *N*,*N*-dimethyl-4-methoxybenzamide, **3b** was obtained as a viscous oil (534 mg, 82%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.04 and 1.07 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 3.02—3.67 (4H, br, 2×$NC\underline{H}_2CH_3$), 3.86 (3H, s, OMe), 4.34 (2H, br s, $COCH_2$), 6.93 (2H, d, J=8.9 Hz, Ar), 7.20—7.36 (4H, m, Ar), 8.00 (2H, d, J=8.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1676, 1610. HR-MS *m/z*: 325.1679 (Calcd for $C_{20}H_{23}NO_3$: 325.1680).

***N*,*N*-Diethyl-2-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]benzamide (3c)** Starting with **2a** and *N*,*N*-dimethyl-3,4-dimethoxybenzamide, **3c** was obtained as colorless powders (584 mg, 82%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.07 (6H, t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 3.03—3.68 (4H, br, 2×$NC\underline{H}_2CH_3$), 3.92 (3H, s, OMe), 3.94 (3H, s, OMe), 4.34 (2H, br s, $COCH_2$), 6.89 (1H, d, J=8.6 Hz, Ar), 7.22—7.36 (4H, m, Ar), 7.54 (1H, d, J=2.0 Hz, Ar), 7.71 (1H, dd, J=8.6, 2.0 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1673, 1614. HR-MS *m/z*: 355.1768 (Calcd for $C_{21}H_{25}NO_4$: 355.1780).

***N*,*N*-Diethyl-2-(2-oxopropyl)benzamide (3d)** Starting with **2a** and *N*,*N*-dimethylacetamide, **3d** was obtained as a viscous oil (376 mg, 81%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.08 and 1.22 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.19 (3H, s, Me), 3.17 (2H, q, J=7.1 Hz, $NC\underline{H}_2CH_3$), 3.40—3.62 (2H, br, $NC\underline{H}_2CH_3$), 3.81 (2H, br s, $COCH_2$), 7.18—7.37 (4H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1717, 1616. HR-MS *m/z*: 233.1426 (Calcd for $C_{14}H_{19}NO_2$: 233.1420).

***N*,*N*-Diethyl-2-(2-oxooctyl)benzamide (3e)** Starting with **2a** and *N*,*N*-dimethylheptanamide, **3e** was obtained as a viscous oil (444 mg, 73%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.87 (3H, t, J=6.6 Hz, $(CH_2)_5C\underline{H}_3$), 1.08 and 1.21 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 1.19—1.35 (6H, m, $(C\underline{H}_2)_3CH_3$), 1.48—1.62 (2H, m, $COCH_2C\underline{H}_2$), 2.47 (2H, t, J=7.4 Hz, $COC\underline{H}_2CH_2$), 3.17 (2H, q, J=7.1 Hz, $NC\underline{H}_2CH_3$), 3.38—3.62 (2H, br, $NC\underline{H}_2CH_3$), 3.77 (2H, br s, $COC\underline{H}_2Ar$), 7.16—7.37 (4H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1710, 1616. HR-MS *m/z*: 303.2218 (Calcd for $C_{19}H_{29}NO_2$: 303.2200).

***N*,*N*-Diethyl-2-(2-cyclohexyl-2-oxoethyl)benzamide (3f)** Starting with **2a** and, *N*,*N*-dimethylcyclohexanecarboxamide, **3f** was obtained as a viscous oil (397 mg, 66%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.08 and 1.21 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 1.13—1.92 (10H, m, CH_2 in cyclohexane), 2.35—2.48 (1H, m, COCH), 3.04—3.26 (2H, br, $NC\underline{H}_2CH_3$), 3.37—3.64 (2H, br, $NC\underline{H}_2CH_3$), 3.87 (2H, br s, $COCH_2$), 7.16—7.37 (4H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1706, 1615. HR-MS *m/z*: 301.2023 (Calcd for $C_{19}H_{27}NO_2$: 301.2040).

***N*,*N*-Diethyl-2-[4-(4-methoxyphenyl)-2-oxobutyl]benzamide (3g)** Starting with **2a** and, *N*,*N*-dimethyl-3-(4-methoxyphenyl)propanamide, **3g** was obtained as a viscous oil (579 mg, 82%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.06 and 1.19 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.69—2.86 (4H, m, $CO(CH_2)_2$), 3.12 (2H, q, J=7.1 Hz, $NC\underline{H}_2CH_3$), 3.37—3.58 (2H, br, $NC\underline{H}_2CH_3$), 3.74 (2H, br s, $COC\underline{H}_2Ar$), 3.77 (3H, s, OMe), 6.80 (2H, d, J=8.6 Hz, Ar), 7.07 (2H, d, J=8.6 Hz, Ar), 7.12—7.36 (4H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1711, 1613. HR-MS *m/z*: 353.1993 (Calcd for $C_{22}H_{27}NO_3$: 353.1990).

***N*,*N*-Diethyl-2-methoxy-6-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]benzamide (3h)** Starting with **2b**[14] and, *N*,*N*-dimethyl-3,4-dimethoxybenzamide, **3h** was obtained as a viscous oil (559 mg, 73%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.02 and 1.08 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.90—3.78 (4H, m, 2×$NC\underline{H}_2CH_3$), 3.81 (3H, s, OMe), 3.92 (3H, s, OMe), 3.93 (3H, s, OMe), 4.23 (2H, s, $COCH_2$), 6.78—6.91 (3H, m, Ar), 7.25 (1H, d, J=7.9 Hz, Ar), 7.54 (1H, d, J=2.0 Hz, Ar), 7.73 (1H, dd, J=8.3, 2.0 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1671, 1610. HR-MS *m/z*: 385.1914 (Calcd for $C_{22}H_{27}NO_5$: 385.1890).

***N*,*N*-Diethyl-2,4-dimethoxy-6-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]-benzamide (3i)** Starting with **2c**[15] and *N*,*N*-dimethyl-3,4-dimethoxybenzamide, **3i** was obtained as a viscous oil (561 mg, 68%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.01 and 1.07 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.93—3.75 (4H, m, 2×$NC\underline{H}_2CH_3$), 3.76 (3H, s, OMe), 3.78 (3H, s, OMe), 3.93 (6H, s, 2×OMe), 4.23 (2H, d, J=1.7 Hz, $COCH_2$), 6.33—6.39 (2H, m, Ar), 6.89 (1H, d, J=8.6 Hz, Ar), 7.55 (1H, d, J=1.7 Hz, Ar), 7.75 (1H, dd, J=8.3, 2.0 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1669, 1602. HR-MS *m/z*: 415.2008 (Calcd for $C_{23}H_{29}NO_6$: 415.2000).

***N*,*N*-Diethyl-2,4-dimethoxy-6-[4-(4-methoxyphenyl)-2-oxobutyl]benzamide (3j)** Starting with **2c**[15] and *N*,*N*-dimethyl-3-(4-methoxyphenyl)-propanamide, **3j** was obtained as a viscous oil (771 mg, 93%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.00 and 1.18 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.75—2.83 (4H, m, $CO(CH_2)_2$), 2.92—3.74 (4H, br m, 2×$NC\underline{H}_2CH_3$), 3.76 (6H, s, 2×OMe), 3.77 (3H, s, OMe), 3.79 (2H, s, $COC\underline{H}_2Ar$), 6.23 (1H, d, J=2.0 Hz, Ar), 6.35 (1H, d, J=2.0 Hz, Ar), 6.80 (2H, d, J=8.6 Hz, Ar), 7.07 (2H, d, J=8.6 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1708, 1605. MS *m/z*: 413 (M^+), 236, 179, 121.

***N*,*N*-Diethyl-2-methoxy-4-methoxymethoxy-6-[2-(3,4-dimethoxyphenyl)-2-oxoethyl]benzamide (3k)** Starting with **2d** and *N*,*N*-dimethyl-3,4-dimethoxybenzamide in DME, **3k** was obtained as colorless crystals (754 mg, 85%). mp: 107—110 °C (AcOEt–*n*-hexane). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.02 and 1.05 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.99—3.72 (4H, m, 2×$NC\underline{H}_2CH_3$), 3.46 (3H, s, OMe), 3.79 (3H, s, OMe), 3.92 (3H, s, OMe), 3.93 (3H, s, OMe), 4.15 and 4.26 (1H each, each d, J=16.5 Hz, $COCH_2$), 5.12 and 5.15 (1H each, each d, J=6.8 Hz, OCH_2O), 6.51 (1H, d, J=2.1 Hz, Ar), 6.52 (1H, d, J=2.1 Hz, Ar), 6.89 (1H, d, J=8.6 Hz, Ar), 7.54 (1H, d, J=2.0 Hz, Ar), 7.71 (1H, dd, J=8.4, 2.0 Hz, Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 12.6, 13.4, 38.2, 42.1, 42.6, 55.4, 56.0×2, 56.1, 94.5, 98.4, 109.8, 110.1, 110.4, 120.7, 123.6, 129.7, 134.3, 148.9, 153.3, 156.5, 158.3, 167.9, 195.7. IR ($CHCl_3$) cm^{-1}: 1670, 1600. HR-MS *m/z*: 445.2097 (Calcd for $C_{24}H_{31}NO_7$: 445.2100). *Anal.* Calcd for $C_{24}H_{31}NO_7$: C, 64.70; H, 7.01; N, 3.14. Found: C, 64.61; H, 7.10; N, 3.18.

***N*,*N*-Diethyl-2-methoxy-6-[2-(3,4-dimethoxyphenyl)-1-oxoethyl]benzamide (10)** Starting with **9** (5 mmol) and *N*,*N*-dimethyl-3,4-(dimethoxybenzene)acetamide (5 mmol), **10** was obtained as a pale yellow viscous oil (208 mg, 11%). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.02 and 1.27 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.98—3.17 (2H, m, $NC\underline{H}_2CH_3$), 3.42—3.73 (2H, m, $NC\underline{H}_2CH_3$), 3.83 (3H, s, OMe), 3.85 (6H, s, 2×OMe), 4.07 and 4.21 (1H each, each d, J=16.2 Hz, $COCH_2$), 6.78—6.84 (3H, m, Ar), 7.05 (1H, t, J=4.6 Hz, Ar), 7.35 (2H, d, J=4.3 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1683, 1617. HR-MS *m/z*: 385.1869 (Calcd for $C_{22}H_{27}NO_5$: 385.1890).

General Procedure for the Synthesis of Isocoumarins (4) and the 1(3*H*)-Isobenzofuranone (11), Synthesis of 3-Phenylisocoumarin (4a) as an Example A solution of **3a** (295 mg, 1 mmol) in AcOH (2 ml) was refluxed under N_2 for 3 h. After evaporation of the solvent, the crude material was purified by column chromatography (AcOEt/*n*-hexane=1/2) and recrystallization from AcOEt–*n*-hexane to give **4a** as colorless needles (202 mg, 91%). mp: 88—89 °C (lit.[16] mp: 87—88 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 6.96 (1H, s, 4-H), 7.40—7.53 (5H, m, Ar), 7.72 (1H, td, J=7.3, 1.3 Hz, Ar), 7.86—7.91 (2H, m, Ar), 8.31 (1H, d, J=7.3 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1723. MS *m/z*: 222 (M^+), 194, 165.

3-(4-Methoxyphenyl)isocoumarin (4b) Starting with **3b**, **4b** was obtained as colorless needles (229 mg, 91%). mp: 114—115 °C (AcOEt–*n*-hexane) (lit.[17] mp: 119—121 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.87 (3H, s, OMe), 6.83 (1H, s, 4-H), 6.97 (2H, d, J=8.9 Hz, Ar), 7.43—7.48 (2H, m, Ar), 7.69 (1H, td, J=7.3, 1.0 Hz, Ar), 7.82 (2H, d, J=8.6 Hz, Ar), 8.29 (1H, d, J=7.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1724. MS *m/z*: 252 (M^+), 224.

3-(3,4-Dimethoxyphenyl)isocoumarin (4c) Starting with **3c**, **4c** was obtained as colorless needles (276 mg, 98%). mp: 120—121 °C (AcOEt–*n*-hexane) (lit.[17] mp: 116 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.94 (3H, s, OMe), 3.99 (3H, s, OMe), 6.85 (1H, s, 4-H), 6.94 (1H, d, J=8.2 Hz, Ar), 7.38 (1H, d, J=2.3 Hz, Ar), 7.45—7.50 (3H, m, Ar), 7.71 (1H, td, J=7.3, 1.3 Hz, Ar), 8.30 (1H, d, J=8.6 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1724. MS *m/z*: 282 (M^+), 254.

3-Methylisocoumarin (4d) Starting with **3d**, **4d** was obtained as colorless needles (139 mg, 87%). mp: 71 °C (*n*-hexane) (lit.[18] mp: 71—72 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 2.29 (3H, s, Me), 6.26 (1H, s, 4-H), 7.32—7.48 (2H, m, Ar), 7.67 (1H, td, J=7.9, 1.3 Hz, Ar), 8.24 (1H, d, J=7.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1720. MS *m/z*: 160 (M^+), 145, 118, 89.

3-Hexylisocoumarin (4e)[19] Starting with **3e**, **4e** was obtained as a colorless oil (229 mg, 99%). bp: 146 °C/3 mmHg. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 0.89 (3H, t, J=6.6 Hz, $(CH_2)_5C\underline{H}_3$), 1.20—1.48 (6H, m, $(C\underline{H}_2)_3CH_3$), 1.63—1.77 (2H, m, $ArCH_2C\underline{H}_2$), 2.53 (2H, t, J=7.6 Hz, $ArC\underline{H}_2$), 6.25 (1H, s, 4-H), 7.32—7.47 (2H, m, Ar), 7.67 (1H, td, J=7.9, 1.3 Hz, Ar), 8.25 (1H, d, J=7.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1718. HR-MS *m/z*: 230.1301 (Calcd for $C_{15}H_{18}O_2$: 230.1310).

3-Cyclohexylisocoumarin (4f) Starting with **3f**, **4f** was obtained as colorless crystals (174 mg, 76%). mp: 94 °C (*n*-hexane) (lit.[19] mp: 91—93 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 1.16—2.19 (10H, m, CH_2 in cyclohexane), 2.23—2.53 (1H, m, $ArC\underline{H}$), 6.23 (1H, s, 4-H), 7.32—7.47 (2H, m, Ar), 7.67 (1H, td, J=7.6, 1.3 Hz, Ar), 8.24 (1H, d, J=7.6 Hz, Ar). IR ($CHCl_3$) cm^{-1}:

1718. HR-MS *m/z*: 228.1165 (Calcd for $C_{15}H_{16}O_2$: 228.1155). *Anal.* Calcd for $C_{15}H_{16}O_2$: C, 78.92; H, 7.06. Found: C, 78.64; H, 7.13.

3-[2-(4-Methoxyphenyl)ethyl]isocoumarin (4g) Starting with **3g**, **4g** was obtained as colorless needles (269 mg, 96%). mp: 87—88 °C (*n*-hexane) (lit.[20] mp: 84—86 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 2.74—3.03 (4H, m, $CO(CH_2)_2$), 3.78 (3H, s, OMe), 6.20 (1H, s, 4-H), 6.83 (2H, d, J=8.6 Hz, Ar), 7.12 (2H, d, J=8.6 Hz, Ar), 7.31—7.69 (3H, m, Ar), 8.26 (1H, d, J=7.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1718. HR-MS *m/z*: 280.1088 (Calcd for $C_{18}H_{16}O_3$: 280.1100). *Anal.* Calcd for $C_{18}H_{16}O_3$: C, 77.12; H, 5.75. Found: C, 77.30; H, 5.78.

8-Methoxy-3-(3,4-dimethoxyphenyl)isocoumarin (4h)[1] Starting with **3h**, **4h** was obtained as colorless crystals (254 mg, 81%). mp: 153—154 °C (AcOEt–*n*-hexane) (lit.[9] mp: 153—154 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.93 (3H, s, OMe), 3.98 (3H, s, OMe), 4.02 (3H, s, OMe), 6.74 (1H, s, 4-H), 6.89—6.94 (2H, m, Ar), 7.01 (1H, d, J=7.9 Hz, Ar), 7.36 (1H, d, J=1.7 Hz, Ar), 7.45—7.63 (2H, m, Ar). IR ($CHCl_3$) cm^{-1}: 1724. MS *m/z*: 312 (M^+), 284.

6,8-Dimethoxy-3-(3,4-dimethoxyphenyl)isocoumarin (4i) Starting with **3i**, **4i** was obtained as colorless crystals (339 mg, 99%). mp: 156—157 °C (AcOEt–*n*-hexane) (lit.[9] mp: 145—146 °C). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.91 (3H, s, OMe), 3.93 (3H, s, OMe), 3.98 (6H, s, 2×OMe), 6.42—6.46 (2H, m, Ar), 6.68 (1H, s, 4-H), 6.91 (1H, d, J=8.6 Hz, Ar), 7.35 (1H, d, J=2.0 Hz, Ar), 7.45 (1H, dd, J=8.4, 2.0 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1713. HR-MS *m/z*: 342.1091 (Calcd for $C_{19}H_{18}O_6$: 342.1100). *Anal.* Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.33; H, 5.30.

6,8-Dimethoxy-3-[2-(4-methoxyphenyl)ethyl]isocoumarin (4j) Starting with **3j**, **4j** was obtained as colorless needles (309 mg, 91%). mp: 147 °C (AcOEt–*n*-hexane) (lit.[21]: reported as an oil). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 2.27 (2H, t, J=7.7 Hz, $C\underline{H}_2CH_2$), 2.95 (2H, t, J=7.7 Hz, $CH_2C\underline{H}_2$), 3.77 (3H, s, OMe), 3.81 (3H, s, OMe), 3.96 (3H, s, OMe), 6.02 (1H, s, 4-H), 6.27 (1H, d, J=2.0 Hz, Ar), 6.42 (1H, d, J=2.0 Hz, Ar), 6.81 (2H, d, J=8.6 Hz, Ar), 7.11 (2H, d, J=8.6 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1710. HR-MS *m/z*: 340.1324 (Calcd for $C_{20}H_{20}O_5$: 340.1310). *Anal.* Calcd for $C_{20}H_{20}O_5$: C, 70.58; H, 5.92. Found: C, 70.59; H, 5.95.

6-Hydroxy-8-methoxy-3-(3,4-dimethoxyphenyl)isocoumarin (4k) A solution of **3k** (1.337 g, 3 mmol) in AcOH (3 ml) and xylene (3 ml) was refluxed under N_2 for 10 h. After evaporation of the solvent, the crude material was dissolved in AcOH (4.5 ml) and refluxed for 5 h. After evaporation of the solvent, the crude drystalls were washed with toluene and recrystallization from DMF–H_2O to give pure **4k** as colorless needles (912 mg, 93%). mp: 272—276 °C (dec.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.82 (3H, s, OMe), 3.86, 3.87 (3H each, s each, OCH_3×2), 6.48 (1H, d, J=2.0 Hz, Ar), 6.52 (1H, d, J=2.2 Hz, Ar), 7.07 (1H, d, J=8.6 Hz, 5′-H), 7.16 (1H, s, 4-H), 7.40 (1H, d, J=2.2 Hz, 2′-H), 7.44 (1H, dd, J=2.0, 8.4 Hz, 6′-H), 10.77 (1H, br s, OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 55.8, 55.9, 56.0, 99.2, 100.6, 100.7, 103.5, 108.3, 111.8, 118.2, 124.4, 142.2, 149.1, 150.6, 152.9, 157.5, 163.4, 164.4. IR (KBr) cm^{-1}: 3256, 1684, 1586, 1260. HR-MS (EI) *m/z*: 328.0945 (Calcd for $C_{18}H_{16}O_6$: 328.0947). *Anal.* Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91, Found: C, 65.57; H, 4.90.

(3*Z*)-7-Methoxy-3-[(3,4-dimethoxyphenyl)methylene]-1(3*H*)-isobenzofuranone (11)[1] Starting with **10**, **11** was obtained as yellow crystals (253 mg, 81%). mp: 185 °C (AcOEt–*n*-hexane). ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 3.93 (3H, s, OMe), 3.97 (3H, s, OMe), 4.03 (3H, s, OMe), 6.34 (1H, s, 8-H), 6.88—6.93 (2H, m, Ar), 7.29 (1H, d, J=7.9 Hz, Ar), 7.39 (1H, dd, J=8.6, 2.0 Hz, Ar), 7.46 (1H, d, J=2.0 Hz, Ar), 7.63 (1H, t, J=7.9 Hz, Ar). IR ($CHCl_3$) cm^{-1}: 1767. MS *m/z*: 312 (M^+), 297, 269.

Ethyl 2-Hydroxy-4-methoxymethoxy-6-methylbenzoate (6) MOMCl (1.3 ml, 18 mmol) was added to a stirred solution of **5** (2.667 g, 14 mmol) and K_2CO_3 (2.442 g, 18 mmol) in acetone (30 ml) under N_2 at 0 °C. The reaction mixture was stirred for 15 h at ambient temperature. H_2O (20 ml) was added to the mixture, and after evaporation of the solvent the products were extracted with AcOEt (20 ml×3). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a residue, which was purified by column chromatography (AcOEt/*n*-hexane=1/30) and recrystallization from *n*-hexane to give **6** as colorless crystals (2.842 g, 87%). mp: 47—48 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.41 (3H, t, J=7.1 Hz, $CH_2C\underline{H}_3$), 2.52 (3H, s, 6-Me), 3.46 (3H, s, OMe), 4.40 (2H, q, J=7.1 Hz, $C\underline{H}_2CH_3$), 5.17 (2H, s, OCH_2O), 6.38 (1H, dq, J=2.6, 0.7 Hz, Ar), 6.49 (1H, d, J=2.4 Hz, Ar), 11.75 (1H, s, OH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.2, 24.4, 56.2, 61.3, 93.8, 101.5, 106.4, 111.7, 143.3, 161.3, 165.2, 171.6. IR ($CHCl_3$) cm^{-1}: 2952 (br), 1645, 1613, 1575. HR-MS *m/z*: 240.0995 (Calcd for $C_{12}H_{16}O_5$: 240.0998). *Anal.* Calcd for $C_{12}H_{16}O_5$: C, 59.99; H, 6.71. Found: C, 60.00; H, 6.69.

Ethyl 2-Methoxy-4-methoxymethoxy-6-methylbenzoate (7) NaH (60% in mineral oil; 4.138 g, 103 mmol) was added to a stirred solution of **6** (16.569 g, 69 mmol) in THF (300 ml) under N_2 at 0 °C. After stirring for 30 min at the same temperature, MeI (12.9 ml, 207 mmol) was added to the reaction mixture and the whole was stirred for 22 h at ambient temperature. H_2O (100 ml) was added to the mixture, and after evaporation of the solvent the products were extracted with AcOEt (100 ml×3). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a residue, which was purified by column chromatography (AcOEt/*n*-hexane=1/10 to 1/5) to give **7** as a colorless oil (17.378 g, 99%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.36 (3H, t, J=7.1 Hz, $CH_2C\underline{H}_3$), 2.28 (3H, s, 6-Me), 3.46 (3H, s, OMe), 3.79 (3H, s, OMe), 4.36 (2H, q, J=7.1 Hz, $C\underline{H}_2CH_3$), 5.16 (2H, s, OCH_2O), 6.45 (1H, d, J=2.0 Hz, Ar), 6.48 (1H, dd, J=2.1, 0.5 Hz, Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 14.2, 19.6, 55.8, 56.0, 60.9, 94.2, 97.8, 109.2, 117.7, 137.8, 157.9, 158.8, 168.1. IR ($CHCl_3$) cm^{-1}: 2945, 1715. HR-MS *m/z*: 254.1151 (Calcd for $C_{13}H_{18}O_5$: 254.1154).

2-Methoxy-4-methoxymethoxy-6-methylbenzoic Acid (8) A mixture of **7** (5.159 g, 20 mmol) and KOH (12.112 g, 216 mmol) in MeOH (75 ml) was refluxed for 12 h. The reaction mixture was cooled to 0 °C and neutralized by adding of 10% HCl aq., and after evaporation of the solvent the products were extracted with Et_2O (100 ml×3). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a crystalline residue, which was purified by recrystallization from AcOEt–*n*-hexane to give **8** as colorless prisms (4.305 g, 94%). mp: 117—119 °C. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.20 (3H, s, 6-Me), 3.38 (3H, s, OMe), 3.74 (3H, s, OMe), 5.20 (2H, s, OCH_2O), 6.49 (1H, d, J=2.0 Hz, Ar), 6.53 (1H, d, J=2.2 Hz, Ar), 12.70 (1H, br s, OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 19.4, 55.9×2, 93.9, 98.1, 109.1, 119.1, 136.4, 157.1, 158.1, 168.8. IR (KBr) cm^{-1}: 3387, 2951, 1717. HR-MS *m/z*: 226.0844 (Calcd for $C_{11}H_{14}O_5$: 226.0841). *Anal.* Calcd for $C_{11}H_{14}O_5$: C, 58.40; H, 6.24. Found: C, 58.61; H, 6.12.

***N*,*N*-Diethyl-2-methoxy-4-methoxymethoxy-6-methylbenzamide (2d)** EDAC (1.457 g, 7.6 mmol) and DMAP (928 g, 7.6 mmol) was added to a stirred solution of **8** (1.420 g, 6.3 mmol) in $CHCl_3$ (11 ml) under N_2. After stirring for 30 min at rt, diethylamine (3.26 ml, 31.5 mmol) was added to the reaction mixture and the whole was stirred for 23 h at rt. H_2O (20 ml) was added to the mixture, and the products were extracted with AcOEt (30 ml×3). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a residue, which was purified by column chromatography (AcOEt/*n*-hexane=2/1) to give **2d** as a pale yellow oil (1.107 g, 63%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.02 and 1.24 (3H each, each t, J=7.1 Hz, 2×$NCH_2C\underline{H}_3$), 2.20 (3H, s, 6-Me), 3.13 (2H, q, J=7.1 Hz, $NC\underline{H}_2CH_3$), 3.36—3.81 (2H, m, $NC\underline{H}_2CH_3$), 3.49 (3H, s, OMe), 3.76 (3H, s, OMe), 5.15 and 5.17 (1H each, each d, J=6.9 Hz, OCH_2O), 6.43 (1H, d, J=2.2 Hz, Ar), 6.49 (1H, dd, J=2.2, 0.5 Hz, Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 12.8, 13,8, 19.0, 38.5, 42.4, 55.4, 56.0, 94.4, 97.5, 109.2, 120.4, 136.5, 156.4, 158.0, 168.3. IR ($CHCl_3$) cm^{-1}: 2953, 1604. HR-MS *m/z*: 281.1625 (Calcd for $C_{15}H_{23}NO_4$: 281.1627).

General Procedure of Demethylation of 4c, 4h, 4i, 4k and 11, Synthesis of Thunberginol A (1a) as an Example BBr_3 (1.0 M in CH_2Cl_2; 3.0 ml, 3.0 mmol) was added to a stirred solution of the isocoumarin **4h** (156 mg, 0.5 mmol) in CH_2Cl_2 (25 ml) under N_2 at −78 °C. The reaction mixture was stirred for 12 h at ambient temperature. H_2O (5 ml) was added to the mixture, and after evaporation of the solvent the products were extracted with AcOEt (40 ml×2). The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give a residue, which was purified by column chromatography (AcOEt) and recrystallization from DMF–H_2O to give **1a** as yellow crystals (124 mg, 92%). mp: 248—249 °C (lit.[1] mp: 240 °C). ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.88 (1H, d, J=8.3 Hz, 5′-H), 6.93 (1H, d, J=8.3 Hz, 7-H), 7.10 (1H, d, J=7.8 Hz, 5-H), 7.23 (1H, s, 4-H), 7.24 (1H, dd, J=8.3, 2.1 Hz, 6′-H), 7.30 (1H, d, J=2.1 Hz, 2′-H), 7.69 (1H, t, J=8.0 Hz, 6-H), 9.32 (1H, s, OH), 9.59 (1H, s, OH), 10.86 (1H, s, OH). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 100.6, 105.2, 112.2, 114.0, 116.0, 116.6, 117.0, 122.3, 137.6, 138.5, 145.6, 147.8, 152.8, 160.4, 165.2. IR (KBr) cm^{-1}: 3412, 1663. MS *m/z*: 270 (M^+), 242, 213.

3-(3,4-Dihydroxyphenyl)isocoumarin (1d)[22] Starting with **4c** (54 mg, 0.19 mmol), **1d** was obtained as pale yellow plates (46 mg, 94%). mp: 236—237 °C (MeOH–H_2O). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.87 (1H, d, J=8.2 Hz, 5′-H), 7.22 (1H, s, 4-H), 7.25 (1H, dd, J=2.3, 8.3 Hz, 6′-H), 7.31 (1H, d, J=2.2 Hz, 2′-H), 7.54 (1H, dt, J=1.1, 7.6 Hz, 5-H), 7.66 (1H, d, J=7.7 Hz, 6-H), 7.82 (1H, dt, J=1.3, 7.6 Hz, 7-H), 8.13 (1H, td, J=0.6, 8.1 Hz, 8-H), 9.43 (2H, br s, 2×OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 99.8, 112.4, 116.2, 117.1, 119.4, 123.0, 126.4, 128.0, 129.0, 135.5, 138.1, 145.8, 147.9, 153.4, 161.7. IR (KBr) cm^{-1}: 3222, 1687, 1599. MS *m/z*: 254 (M^+), 226, 89, 76, 63. *Anal.* Calcd for $C_{15}H_{10}O_4 \cdot 1/2H_2O$: C, 68.44; H, 4.21. Found: C, 68.37; H, 4.34.

8-Hydroxy-3-(3,4-dihydroxyphenyl)-6-methoxyisocoumarin (1e) Starting with **4i**, **1e** was obtained as pale yellow crystals (145 mg, 97%). mp: 231—232 °C (DMF–H_2O). ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 3.87 (3H, s, OMe), 6.51 (1H, d, J=2.3 Hz), 6.67 (1H, d, J=2.3 Hz), 6.87 (1H, d, J=8.3 Hz), 7.15 (1H, s), 7.21 (1H, dd, J=8.3, 2.0 Hz), 7.26 (1H, d, J=2.0 Hz), 9.22—9.78 (2H, br, 2×OH), 10.96 (1H, br s, OH). IR (KBr) cm^{-1}: 3335, 1676. MS m/z: 300 (M^+), 272. *Anal.* Calcd for $C_{16}H_{12}O_6 \cdot 3/5H_2O$: C, 61.78; H, 4.27. Found: C, 61.78; H, 4.24.

Thunberginol B (1b) Starting with **4k**, **1b** was obtained as pale yellow crystals (240 mg, 84%). mp: 288—293 °C (MeOH–H_2O). (lit.[9] mp: 281—285 °C, lit.[1] mp: 244 °C). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.30 (1H, d, J=2.0 Hz, 5-H), 6.47 (1H, d, J=2.0 Hz, 7-H), 6.83 (1H, d, J=8.4 Hz, 5′-H), 7.09 (1H, s, 4-H), 7.20 (1H, dd, J=8.2, 2.2 Hz, 6′-H), 7.25 (1H, d, J=2.2 Hz, 2′-H), 9.28 (1H, br s, OH), 9.55 (1H, br s, OH), 10.84 (1H, br s, OH), 10.92 (1H, br s, OH). ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 98.0, 100.6, 101.4, 103.2, 112.2, 116.0, 117.0, 122.4, 140.1, 145.6, 147.7, 152.8, 162.6 165.0, 165.6. IR (KBr) cm^{-1}: 3332, 3149, 2834, 1667, 1614, 1521, 1241. HR-MS m/z: 286.0478 (Calcd for $C_{15}H_{10}O_6$: 286.0477).

Thunberginol F (1c) Starting with **11**, **1c** was obtained as yellow crystals (132 mg, 98%). mp: 220—240 °C (AcOEt–*n*-hexane) (lit.[1] mp: 242—243 °C). ^{1}H-NMR (270 MHz, DMSO-d_6) δ: 6.62 (1H, s, 8-H), 6.79 (1H, d, J=8.3 Hz, 5′-H), 6.91 (1H, d, J=8.2 Hz, 6-H), 7.02 (1H, br d, J=8.3 Hz, 6′-H), 7.38 (1H, br s, 2′-H), 7.41 (1H, d, J=7.9 Hz, 4-H), 7.58 (1H, t, J=7.9 Hz, 5-H), 9.25 (2H, br, 2×OH), 11.02 (1H, br, OH). IR (KBr) cm^{-1}: 3204, 1745. MS m/z: 270 (M^+), 213, 168.

Bioassay Method As a marker of the degranulation of RBL-2H3 cells, release of β-hexosamindase into the medium was determined as described previously.[5,23] Briefly, RBL-2H3 cells [Cell No. JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) and penicillin (100 units/ml) and streptomycin (100 μg/ml) were seeded into 24-well multiplates at the density of 2×10^5 cells per well and were incubated with anti-dinitrophenyl (DNP) IgE antibody (0.45 μg/ml, monoclonal anti-DNP, Sigma) for sensitization of the cells. Then, the cells were washed twice with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, and 40 mM NaOH, pH 7.2) supplemented with 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA) and incubated in 160 μl of buffer for 10 min at 37 °C. Then, cells were added with 20 μl of test sample solution, and were stimulated with 20 μl of dinitrophenylated bovine serum albumin (DNP-BSA)[4] (final conc. 10 μg/ml) as an antigen for 10 min. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 μl) was transferred into a 96-well microplate and incubated with 50 μl of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to incubation buffer (final DMSO conc. was 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test sample was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{inhibition (\%)} = [1-(T-B-N)/(C-N)]\times 100$$

Control (C): DNP-BSA (+), test sample (−);
Test (T): DNP-BSA (+), test sample (+);
Blank (B): DNP-BSA (−), test sample (+);
Normal (N): DNP-BSA (−), test sample (−).

Under the same conditions, IC_{50} values of reference compounds, tranilast and ketotifen fumarate were 282 and 158 μM as reported previously.[23] In order to clarify that the anti-allergic effects of samples were due to the inhibition of degranulation, but not the false positive by the inhibition of β-hexosaminidase activity, the following assay was carried out.[23] Briefly, the cell suspension of PBS was sonicated, and the solution was then centrifuged. The supernatant was diluted with Siraganian buffer and adjusted to equal the enzyme activity of the degranulation tested above. The enzyme solution (45 μl) and test sample solution (5 μl) were transferred into a 96-well microplate and incubated with 50 μl of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μl of the stop solution and the absorbance was measured using a microplate reader at 405 nm.

Each inhibition (%) was represents the mean±S.E.M. (n=4). A one-way analysis of variance followed by Dunnett's test for multiple comparisons was used for the statistical analysis. Probability (p) values of less than 0.05 were considered significant.

Acknowledgements We are grateful for financial support in part by the Frontier Research Program and the 21st Century COE Program 'Development of Drug Discovery Frontier Integrated from Traditional to Proteome' from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and a Grant-in-Aid for the Promotion of the Advancement of Education and Research in Graduate Schools in Subsidies for ordinary expenses of private schools from the Promotion and Mutual Aid Corporation for Private Schools.

References

1) Yoshikawa M., Harada E., Naitoh Y., Inoue K., Matsuda H., Shimoda H., Yamahara J., Murakami N., *Chem. Pharm. Bull.*, **42**, 2225—2230 (1994).
2) Yamahara J., Matsuda H., Shimoda H., Wariishi N., Yagi N., Murakami N., Yoshikawa M., *Folia Pharmacol. Jpn.*, **105**, 365—379 (1995).
3) Yoshikawa M., Matsuda H., Shimoda H., Shimada H., Harada E., Naitoh Y., Miki A., Yamahara J., Murakami N., *Chem. Pharm. Bull.*, **44**, 1440—1447 (1996).
4) Matsuda H., Shimoda H., Yoshikawa M., *Bioorg. Med. Chem.*, **7**, 1445—1450 (1999).
5) Wang Q., Matsuda H., Matsuhira K., Nakamura S., Yuan D., Yoshikawa M., *Biol. Pharm. Bull.*, **30**, 388—392 (2007).
6) Matsuda H., Wang Q., Matsuhira K., Nakamura S., Yuan D., Yoshikawa M., *Phytomedicine*, **15**, 177—184 (2008).
7) Ohta S., Kamata Y., Inagaki T., Masuda Y., Yamamoto S., Yamashita, M., Kawasaki I., *Chem. Pharm. Bull.*, **41**, 1188—1190 (1993).
8) Snieckus V., *Chem. Rev.*, **90**, 879—933 (1990).
9) Rossi R., Carpita A., Bellina F., Stabile P., Mannina L., *Tetrahedron*, **59**, 2067—2081 (2003).
10) Gramatica P., Gianotti M. P., Speranza G., Manitto P., *Heterocycles*, **24**, 743—750 (1995).
11) Cheong H., Choi E.-J., Yoo G.-S., Kim K.-M., Ryu S.-Y., *Planta Med.*, **64**, 577—578 (1998).
12) Kaul S., Hoffmann A., *ALTEX.*, **18**, 55—58 (2001).
13) Dorman G., Prestwich G. D., *Trends Biotechnol.*, **18**, 64—77 (2000).
14) Watanabe M., Sahara M., Kubo M., Furukawa S., Billedeau R. J., Snieckus V., *J. Org. Chem.*, **49**, 742—747 (1984).
15) Superchi S., Pini D., Salvadori P., Marinelli F., Rainaldi G., Zanelli U., Nuti-Ronchi V., *Chem. Res. Toxicol.*, **6**, 46—49 (1993).
16) Larock R. C., Varaprath S., Lau H. H., Fellows C. A., *J. Am. Chem. Soc.*, **106**, 5274—5284 (1984).
17) Bradsher C. K., Wallis T. G., *J. Org. Chem.*, **43**, 3817—3820 (1978).
18) Tirodkar R. B., Usgaonkar R. N., *J. Ind. Chem. Soc.*, **46**, 935—944 (1969).
19) Ogawa Y., Maruno M., Wakamatsu T., *Heterocycles*, **41**, 2587—2599 (1995).
20) Rama N. H., Iqbal R., Zamani K., Saeed A., Choudhary M. I., *Indian J. Chem.*, **37B**, 480—483 (1998).
21) Saeed A., Rama N. H., Arfan M., *J. Heterocycl. Chem.*, **40**, 519—522 (2003).
22) Ahmad H. B., Rama N. H., Hussain M., Hussain M. T., Qasim M. M., Hameed S., Malana M. A., Malik A., *Indian J. Chem.*, **42B**, 611—615 (2003).
23) Matsuda H., Tewtrakul S., Morikawa T., Nakamura A., Yoshikawa M., *Bioorg. Med. Chem.*, **12**, 5891—5898 (2004).