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Development of 6-Arylcoumarins as Nonsteroidal Progesterone Antagonists. Structure-activity Relationships and Fluorescence Properties

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Abstract

Progesterone is involved in multiple physiological processes, including female reproduction, via binding to the progesterone receptor (PR). We have developed 6-arylcoumarins such as **5** and **6** as non-steroidal PR antagonists with receptor-binding-dependent fluorescence. In this study, we investigated the structure-activity relationships and fluorescence properties of coumarin derivatives bearing a heterocyclic aromatic moiety. Among these derivatives, **7c** (IC_{50}: 34 nM) and **10b** (IC_{50}: 24 nM) showed more potent PR-antagonistic activity than lead compounds **5** (IC_{50}: 500 nM) and **6** (IC_{50}: 65 nM) in alkaline phosphatase (AP) assay. Compound **9b** showed solvent-dependent fluorescence intensity, exhibiting strong fluorescence in the presence of PR LBD only in buffer solution. On the other hand, **10b** showed a solvent-dependent shift of the fluorescence maximum wavelength in the presence of PR LBD. These results indicate that 6-arylcoumarin will be a useful scaffold for PR antagonists and fluorescent probes targeting PR.

Keywords

progesterone, antagonist, fluorescent ligand, coumarin

1. Introduction

Progesterone (**1**, Fig. 1) has significant roles in various physiological processes, including uterine cell proliferation and differentiation, the ovulation cycle, and mammary gland growth and differentiation in females.[1-4] Functions of progesterone (**1**) in the cardiovascular,[5] immune,[6] and nervous systems[7] have also been reported. These activities of progesterone (**1**) are mediated by the progesterone receptor (PR), which is a ligand-dependent transcriptional factor belonging to the nuclear receptor superfamily.[8] Various synthetic PR agonists and antagonists have been developed for clinical use.[9,10] For example, PR antagonist mifepristone (**2**) has been clinically used as an abortifacient.[11] Recent studies suggested that PR antagonists might be effective in the treatment of endometriosis,[12] uterine leiomyoma,[13] and breast cancer.[14,15] However, PR antagonists with a steroidal skeleton, such as **2**, show cross-activity toward other steroid hormone receptors,[16] and often have significant adverse effects. Therefore, nonsteroidal PR-antagonists have been developed, such as **3a** and **4a**.[17-21] Interestingly, even minor structural modification can change the biological functions of these compounds; for example, compounds **3b**, **3c**, and **4b** acted as PR agonists, not PR antagonists.

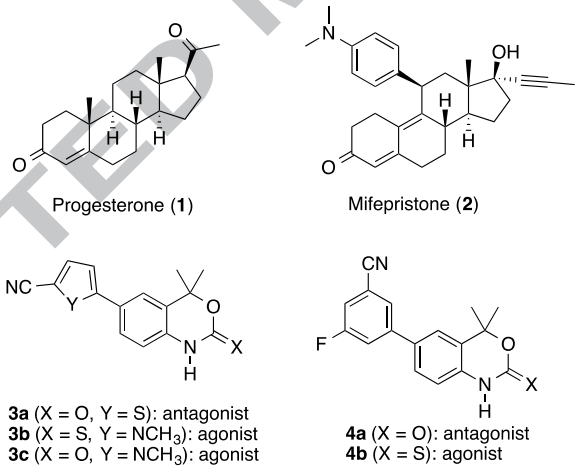
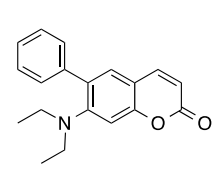


Figure 1. Structures of progesterone (**1**), mifepristone (**2**), and typical nonsteroidal PR ligands, **3** and **4**.

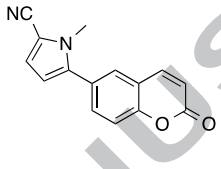
We previously reported 6-arylcoumarins as novel nonsteroidal PR antagonists.[22] Compound **5** exhibited potent PR antagonistic activity in alkaline phosphatase (AP) assay with T47D human breast carcinoma cells (IC_{50}: 0.50 μM), and showed high binding affinity towards recombinant hPR ligand-binding domain (LBD) (IC_{50}: 1.42 μM). Compound **5** also has possible application as a fluorescent probe for PR, since its fluorescence is increased in the presence of PR LBD, though the fluorescence quantum yield (0.744 in methylene chloride) was moderate and depended on the solvent properties. Further, compound **5** has binding affinity for other steroid hormone receptors,

such as hGR (IC_{50}: 3.54 μM) and hAR (IC_{50}: 10.0 μM). Therefore, novel compounds with higher fluorescence quantum yield and greater PR selectivity are needed for development of fluorescent probes for PR. Among the 6-arylcoumarin derivatives synthesized here, we found that some compounds bearing a pyrrole or thiophene, but not furane, as 6-aryl group, showed potent PR antagonistic activity.[20] For example, compound **6** showed more potent PR antagonistic activity (IC_{50}: 0.065 μM) than **5** in AP assay. Although the binding affinity of **6** to recombinant hPR LBD is very low (IC_{50}: 15.2 μM), **6** did not bind to other steroid hormone receptors.



5

IC_{50} (AP assay) 0.50 μM
IC_{50} (PR binding) 1.42 μM

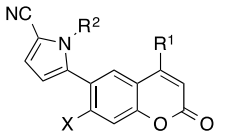


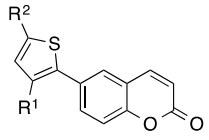
6

IC_{50} (AP assay) 0.065 μM
IC_{50} (PR binding)15.2 μM

Figure 2. Structures of PR-antagonistic coumarin derivatives

From a consideration of our previous work on coumarin derivatives with PR antagonistic activity, together with the structures of known PR ligands,[17-21] we expected that linkage of coumarin to a five-membered heterocyclic ring would be favorable for high PR-antagonistic activity. Further, introduction of appropriate substituents on the coumarin ring, especially at the 7 position or on the five-membered heterocyclic ring, should alter the PR-binding affinity and the fluorescence properties.[23] Therefore, in this study, we examined the structure–PR antagonist activity relationships of coumarin derivatives bearing pyrrole (**7**, **9** – **11**) or thiophene (**8**) at the 6 position (Figure 3).





8a -e

7a - l	X = H, R^1 = H
9a - e	X = NEt_2, R^1 = H
10a - h	X = OMe, R^1 = Me
11	X = OH, R^1 = Me

Figure 3. Structures of synthesized derivatives of **6**

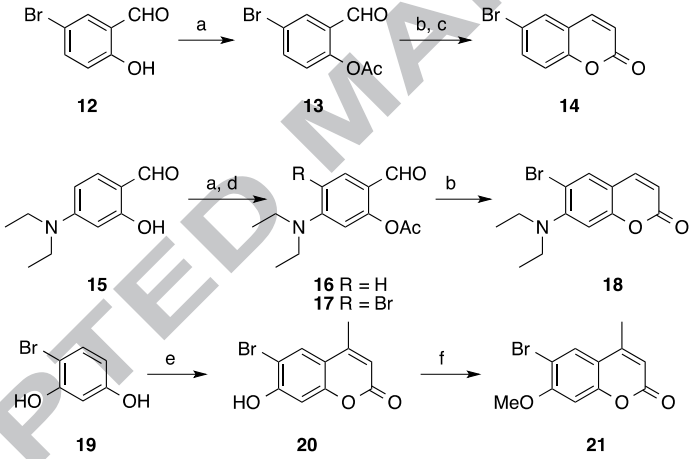
2. Results and discussion

2.1. Synthesis of coumarin derivatives

In order to clarify the substituent effects on the 6-pyrrole ring of **6**, compounds **7a** – **l** with various *N*-substituents and compounds **8a** – **e** bearing a substituted thiophene instead of the pyrrole ring of **6** were synthesized. Then, the effects of 7-substituents, whose steric and electronic effects would

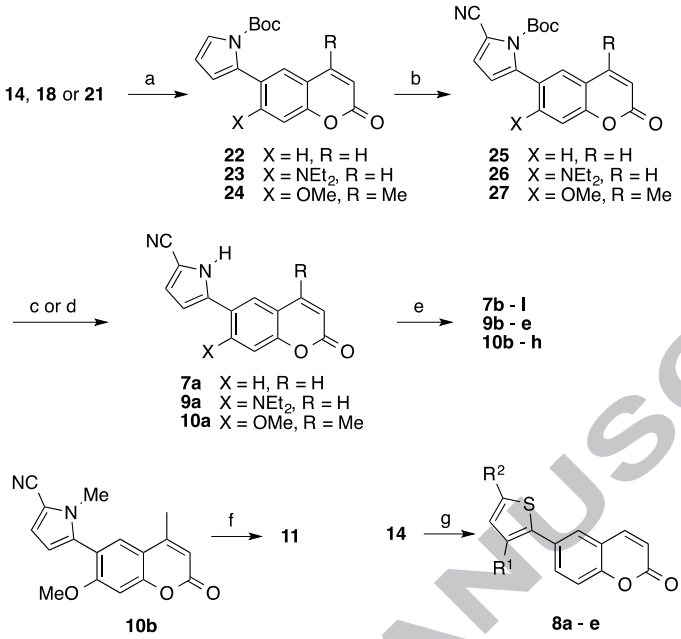
influence the properties of 6-aryl groups on the coumarin ring, were examined by synthesis of compounds **9a** – **e**, **10a** – **h**, and **11** (Figure 3). In the case of compounds **10** and **11**, 4-methylcoumarin derivatives were designed and synthesized for synthetic convenience.

6-Arylcoumarins **7** – **11** were prepared by Suzuki-Miyaura cross-coupling reaction of the corresponding 6-bromocoumarin with arylboronic acid derivatives.[24] Synthesis of 6-bromocoumarins **14**, **18** and **21** is illustrated in Scheme 1. The Stille-Gennari modification[25] of Horner-Wadsworth-Emmons olefination of 2-acetoxy-5-bromobenzaldehyde (**13**), prepared by acetylation of **12** (quant), afforded a mixture of 6-bromocoumarin and acyclic methyl cinnnamate derivative. Treatment of the mixture with hydrochloric acid in methanol gave 6-bromocoumarin in 97% yield (two steps). 4-Diethylaminosalicylic acid was acetylated (quant), followed by bromination (49%) to afford **17**. The Stille-Gennari modification of Horner-Wadsworth-Emmons olefination of **17** afforded **18** in 48% yield. Derivative **21** with a 6-methoxy group was prepared by reaction of 4-bromoresorcinol with ethyl acetoacetate (75%), followed by *O*-methylation (quant).



Scheme 1. Synthesis of 6-bromocoumarins **14**, **18** and **21**. Reagents and conditions: (a) Ac_2O, pyridine, CH_2Cl_2; (b) $(CF_3CH_2O)_2P(O)CH_2COOMe$, KHMDS, 18-crown-6, THF, -78°C then rt; (c) 4 M HCl, MeOH. 85°C; (d) NBS, THF, 0°C; (e) ethyl acetoacetate, H_2SO_4; (f) K_2CO_3, MeI, DMF.

The Suzuki-Miyaura cross-coupling reactions of **14**, **18**, and **21** with *N*-Boc-pyrrole-2-boronic acid in the presence of CsF and $PdCl_2$(dppf) in DMF afforded 6-(*N*-Boc-2-pyrrolyl)coumarins **22** – **24**, respectively. Reaction of **22** – **24** with chlorosulfonyl isocyanate gave 5-cyano derivatives **25** – **27**, respectively.[21] After deprotection of the Boc group by heating in water (for **25** and **27**) or with hydrochloric acid in ethanol (for **26**), various *N*-substituents were introduced to obtain **7**, **9**, and **10**. 6-Hydroxycoumarin derivative **11** was prepared by demethylation of **10b** using boron tribromide (44%). 6-Thiophenylcoumarins **8** were also prepared from **14** by Suzuki-Miyaura cross-coupling reaction with substituted thiophene boronic acid or its pinacol ester.

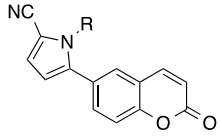


Scheme 2. Synthesis of 6-arylcoumarins **7** - **11**. Reagents and conditions: (a) *N*-Boc-pyrrole-2-boronic acid, CsF, $PdCl_2$(dppf), DMF, 60°C; (b) $ClSO_2NCO$, THF, -78°C, then DMF, rt; (c) H_2O, 100°C (for **25** and **27**); (d) 8% HCl in EtOH, 50°C (for **26**); (e) NaH, alkyl halide, DMF; (f) BBr_3, CH_2Cl_2, -78°C then rt; (g) substituted thiophene-2-boronic acid or its pinacol ester, CsF, $PdCl_2$(dppf), DMF, 60°C.

2.2. PR-antagonistic activity

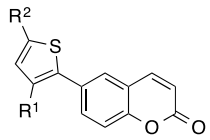
The PR-agonistic and antagonistic activities of the coumarin derivatives were evaluated by means of alkaline phosphatase (AP) assay[22] using T-47D human breast carcinoma cell line. None of the test compounds alone induced alkaline phosphatase expression (data not shown), which means that they did not act as PR agonists. PR-antagonistic activity of the synthesized compounds was examined in the presence of 1 nM progesterone. First, the effect of the *N*-substituent on the pyrrole ring of compound **6** was examined (Table 1). Removal of the *N*-methyl group of **6**, yielding compound **7a**, decreased the activity, while replacement of the *N*-methyl group with a moderate-sized substituent, such as *n*-hexyl (**7c**), cyclohexylmethyl (**7d**), or benzyl (**7f**), resulted in retention of potent PR-antagonist activity. Introduction of a polar functional group, such as cyano (**7e** and **7g**), nitro (**7h**), or fluorine (**7i – k**), decreased the activity. Thus, a moderate-sized hydrophobic *N*-substituent on the pyrrole ring is important for PR-antagonistic activity. Next, we examined the activity of 6-thiophenylcoumarin derivatives. All thiophene derivatives exhibited lower activity, independent of the substituents on the thiophene ring. For example 5-cyanothiphen-2-ylcoumarin (**8d**) has an IC_{50} value of 0.14 μM, which is comparable to that of compound **7a** without the *N*-substituent, and a half as potent as compound **6** with an *N*-methyl group. The result also supports the importance of the *N*-substituent of 6-pyrrolylcoumarin derivatives.

Table 1. PR-antagonistic activity of 6-pyrrolylcoumarins **7**



Compound	Substituent R	T47D IC_{50} (μM)
5		0.50
6		0.065
7a	H	0.14
7b	Et	0.22
7c	*n*-C_6H_{13}	0.034
7d	CH_2-c-C_6H_{11}	0.031
7e	CH_2CN	15.8
7f	CH_2Ph	0.056
7g	CH_2Ph-*p*-CN	0.29
7h	CH_2Ph-*p*-NO_2	0.45
7i	CH_2Ph-*p*-F	0.21
7j	CH_2Ph-3,5-F_2	0.16
7k	$CH_2C_6F_5$	0.19
7l	CH_2-2-naphthyl	0.29

Table 2. PR-antagonistic activity of 6-thiophenylcoumarins **8**

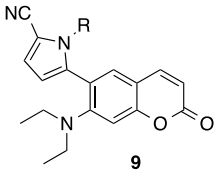


Compound	Substituent R^1	R^2	T47D IC_{50} (μM)
8a	Me	H	0.15
8b	Cl	H	0.23
8c	H	Me	0.44
8d	H	CN	0.14
8e	H	$COCH_3$	0.34

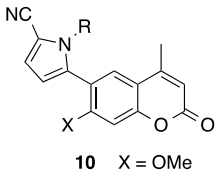
Finally, the effect of 7-substituents on the coumarin ring was examined. Besides compounds **7** lacking a 7-substituent, derivatives with a 7-diethylamino group (compounds **9**), 7-methoxy group (compounds **10**) and 7-hydroxy group (compound **10**) were synthesized. 7-Diethylaminocoumarin derivatives having a rather small *N*-substituent on the pyrrole ring showed moderate activity, and a larger *N*-substituent decreased the activity. 7-Methoxycoumarin derivatives with an *N*-methyl (compound **10b**) or *N*-ethyl (compound **10c**) group showed potent PR-antagonistic activity with a lower IC_{50} value (**10a**: 0.024 μM, **10b**: 0.0384 μM) than that of compound **6** (IC_{50}: 0.065 μM) lacking a 7-substituent. Introduction of a bulky *N*-substituent on the pyrrole ring of 7-methoxycoumarin derivatives decreased the activity, which is different from the case of 7-unsubstituted coumarin derivatives. Coumarin derivative **11** with a 7-hydroxy group showed weak activity, compared to the 7-methoxy derivative **10b**. Thus, the PR-antagonistic activity of 6-pyrrolylcoumarin depended on the hydrophobicity and bulkiness of the 7-substituent on the

coumarin ring and the *N*-substituent on the pyrrole ring.

Table 3. PR-antagonistic activity of 7-substituted 6-pyrrolylcoumarins **9 - 11**



9



10 X = OMe
11 X = OH

Compound	Substituent (R)	T47D IC_{50} (μM)
9a	H	0.12
9b	Me	0.24
9c	Et	0.25
9d	CH_2CN	0.85
9e	CH_2Ph	0.58
10a	H	0.26
10b	Me	0.024
10c	Et	0.038
10d	*n*-C_6H_{13}	0.18
10e	CH_2-c-C_6H_{11}	0.55
10f	CH_2Ph	0.23
10g	CH_2Ph-*p*-CN	0.48
10h	CH_2Ph-*p*-F	0.57
11	Me	0.3

The PR-binding affinity of selected compounds was evaluated by means of competitive binding assay using the PR LBD and [^{3}H]progesterone (Table 4). Our previous study showed that 6-aryl derivatives with a 7-diethylamino group such as **5** had high PR-binding affinity, while 7-unsubstituted derivatives, such as **6** with higher potency in AP assay, had low PR-binding affinity.[22] In this study, we found that introduction of 7-diethylamino or 7-methoxy group into the structure of **6** resulted in a dramatic improvement of the PR-binding affinity; the IC_{50} values of compounds **9b** (1.36 μM) and **10b** (0.99 μM) are comparable to or better than that of **6** (1.42 μM). Among 7-unsubstituted derivatives, compounds **7c** and **7f**, which showed potent PR-antagonistic activity in AP assay, had also higher PR-binding affinity than **6**.

Table 4. PR-Binding affinity of 6-pyrrolylcoumarins

Compound	Binding IC_{50}[a] (μM)
5	1.42
6	15.2
7c	0.16
7f	0.79
9b	1.36
10b	0.99
11	15.7

[a][^{3}H]Progesterone concentration was 4 nM.

Some PR ligands can bind to other steroid hormone receptors. Among our previously reported 6-arylcoumarins, compound **5** binds to androgen receptor (AR) and glucocorticoid receptor (GR), but compound **6** does not. To examine the PR selectivity of the present compounds, we focused on **9b** and **10b**, which have the same 6-aryl group as **6**, and examined their activity towards other nuclear receptors. Androgenic activity was examined in terms of the effect on the growth of androgen-dependent SC-3 cells.[26] Neither **9b** nor **10b** alone affected the growth of SC-3 cells. AR-antagonistic activity was evaluated in terms of the effect of test compounds on the cell growth induced by 1 nM dihydrotestosterone (DHT). Compound **10b** showed AR-antagonistic activity with similar potency to the well-known AR antagonist, hydroxyflutamide (Figure 4), while **9b** showed AR-inhibitory activity only at 10^{-5} M. The GR-binding affinity of **9b** and **10b** was examined by competitive binding assay with hGR and [^{3}H]dexamethasone.[22] Only compound **9b** showed binding affinity, with an IC_{50} value of 10.0 μM, which is weaker than compound **5** (IC_{50}: 3.54 μM). Neither of the compounds showed mineralocorticoid receptor (MR)-binding affinity in competitive binding assay using the rMR and [^{3}H]aldosterone (data not shown).[22] Thus, the PR selectivity of **9b** and **10b** was improved, although some cross-reactivity remained.

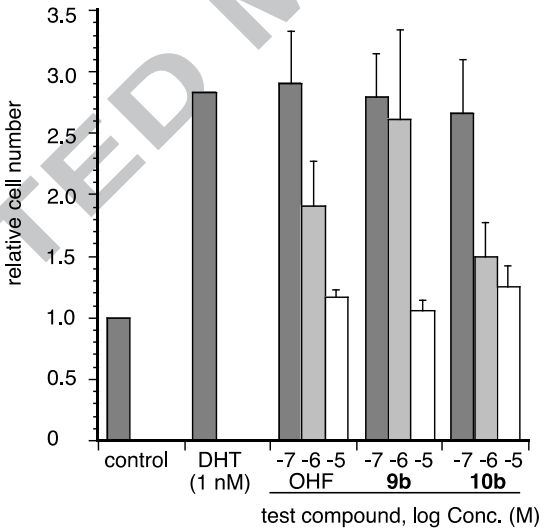


Figure 4. AR-antagonistic activity in SC-3 cell growth assay. Vertical scale is the relative cell number when the cell number of the control (solvent only) is taken as 1. OHF: Hydroxyflutamide.

Table 5. GR- and MR-Binding affinity of 6-pyrrolylcoumarins

Compound	IC_{50} (μM)[a]	
	hGR	rMR
5	3.54	ND
6	ND	ND
9b	10.0	ND
10b	ND	ND

[a]ND: no detectable binding

2.3. Fluorescence properties

Compound **5** exhibited solvent-dependent fluorescence; that is, strong fluorescence was observed in methylene chloride (quantum yield, Φ_{fl}: 0.744), while almost no fluorescence was seen in polar solvents such as methanol (Φ_{fl}: 0.007) and 10 mM sodium phosphate buffer (pH 7.4, Φ_{fl}: 0.007).[22] The fluorescence intensity of **5** in the buffer increased upon addition of PR LBD. Solvent dependency of the coumarin derivative without 7-substituent is less than compound **5** with 7-diethylamino group. Therefore, we examined the flouresnt properties of **9b** with 7-methoxy group and **10b** with 7-hydroxy group.

Solvent-dependent florescence was also observed with compound **9b** (Figure 5a, Table 6): the fluorescence intensity of **9b** was strong in methylene chloride (Φ_{fl}: 0.892), weak in polar solvents such as acetone (Φ_{fl}: 0.125) and acetonitrile (Φ_{fl}: 0.057), and almost absent in methanol (Φ_{fl}: 0.006) and 10 mM sodium sodium phosphate buffer (pH 7.4, Φ_{fl}: 0.004). The florescence quantum yield of **9b** (Φ_{fl}: 0.892) in methylene chloride was larger than that of **5** (Φ_{fl}: 0.744). Interestingly, the fluorescence of compound **10b** showed a different type of solvent-dependency: the fluorescence intensity showed little dependence on the solvent, except in buffer, but the fluorescence maximum wavelength shifted in the range of 419 (methylene chloride) to 466 nm (methanol). Previously, we have reported some 6-arylcoumarin sensors in which an analyte recognition site was introduced at the 6-aryl group. In these sensors, the mode of fluorescence change induced by binding to the analyte was a change of fluorescence intensity for compounds with a 7-diethylamino group, and a change of fluorescence maximum wavelength for compounds with a 7-methoxy group, which is similar to solvent-dependency of compounds **9b** and **10b**.[23] Although the quantum yield of **10b**-type compounds should be improved, fluorescent ligands with different modes of fluorescence change would be useful for development of fluorescent probes for analysis of PR functions. Finally, the fluorescence spectra of **9b** in the presence of PR LBD were examined (Figure 6). Compound **9b** alone did not show fluorescence in buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 8.0), but addition of PR LBD increased the fluorescence intensity. The increase of florescence intensity of **9b** upon addition of PR LBD (500 nM) was larger than that of **5**,[20] probably due to both larger quantum yield and higher PR binding affinity of **9b**, compared to those of **5**. Thus, compound **9b** should be a more sensitive sensor for PR, compared to previously reported **5**.

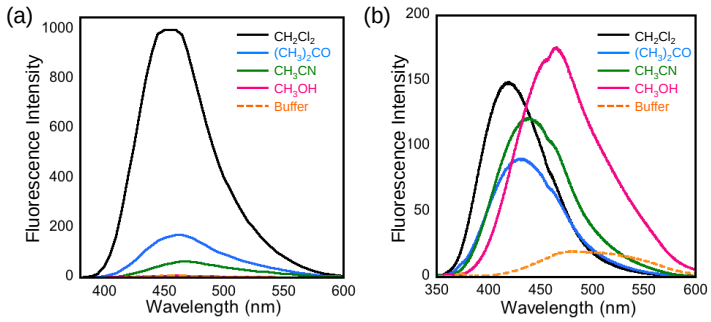


Figure 5. Fluorescence spectra of (a) **9b** and (b) **10b** (2 μM) in various solvents. Buffer is 10 mM sodium phosphate buffer (pH 7.4). Each solvent contained 0.2% DMSO as a cosolvent. Excitation wavelengths were 350 nm for **9b** and 330 nm for **10b**.

Table 6. Fluorescence properties of coumarin derivatives **9b** and **10b**

Compound		Solvent[a]				
		CH_2Cl_2	$(CH_3)_2CO$	CH_3CN	CH_3OH	buffer[b]
9b	Em_{max}, nm	452	464	467	460	470
	Φ_{fl}[c]	0.892	0.125	0.057	0.006	0.004
10b	Em_{max}, nm	419	430	437	466	486
	Φ_{fl}[c]	0.139	0.056	0.076	0.113	0.015

[a]Each solvent contained 0.2% DMSO as a cosolvent.

[b]Buffer is 10 mM sodium phosphate buffer (pH 7.4).

[c]Fluorescence quantum yields (Φ_{fl}) were determined using quinine sulfate (0.577) in 0.1 M sulfuric acid as a standard.

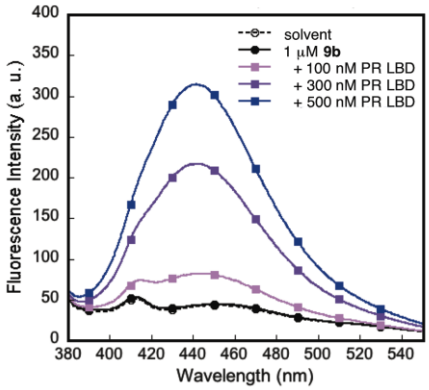


Figure 6. Fluorescence spectra of **9b** (1 μM) in the presence of PR LBD in buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 8.0). Excitation wavelength was 360 nm.

3. Conclusion

We designed and synthesized novel coumarins bearing an aromatic heterocycle as nonsteroidal PR antagonists by using **5** and **6** as lead compounds. Some compounds, such as **7c** and **10b**, showed

more potent PR-antagonistic activity than the lead compounds in AP assay and PR-binding experiments. None of them showed PR-agonistic activity in AP assay, and therefore 6-arylcoumarin should be a useful scaffold for development of PR antagonists, since some of the known PR ligands can act as either agonists or antagonists depending on their substituents. There were two modes of fluorescence change in various solvents, that is, change of fluorescence intensity (**9b**) and change of fluorescence maximum wavelength (**10b**). Compound **9b** showed no fluorescence in buffer alone, but showed strong fluorescence in the presence of PR LBD, and therefore is a sensor candidate for PR functions. The change of fluorescence maximum wavelength exhibited by **10b** would be a useful property for a PR sensor, since the compound could be employed for ratiometric measurement, although the quantum yield would need to be improved. Our findings here should contribute to the development of novel nonsteroidal PR ligands and sensors.

4. Experimental

4.1. Chemistry

All reagents were purchased from Sigma-Aldrich Chemical Co., Tokyo Kasei Kogyo Co., Wako Pure Chemical Industries, and Kanto Kagaku Co., Inc. Silica gel for column chromatography was purchased from Kanto Kagaku Co., Inc. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL ECS 400 spectrometer or a Bruker 600 spectrometer. Mass spectral data was obtained on a Bruker Daltonics microTOF-2focus or MStation JMS-700 in the positive ion detection mode.

4.2. Synthesis

4.2.1. Synthesis of 2-acetoxy-5-bromobenzaldehyde (13)

Acetic anhydride (11 mL) was added to a suspension of **12** (5.01 g, 24.9 mmol) in dry pyridine (20 mL) and dry methylene chloride (60 mL) at room temperature. After 1 h, the solvents were removed in vacuo to afford **13** (5.76 g, 95%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.1 (1 H, s), 8.00 (1 H, d, *J* = 2.8 Hz), 7.73 (1 H, dd, *J* = 2.3, 8.7 Hz), 7.10 (1 H, d, *J* = 8.7 Hz), 2.39 (3 H, s); ^{13}C NMR (150MHz, $CDCl_3$) δ 187.3, 169.0, 150.7, 138.1, 133.6, 129.4, 125.5, 119.9, 20.9.

4.2.2. Synthesis of 5-bromocoumarin (14)

Potassium bis(trimethylsilyl)amide (0.5 M solution in toluene, 34 mL, 17.0 mmol) was added to a solution of bis(2,2,2-trifluoromethyl)(methoxycarbonylmethyl)phosphonate (5.58 g, 17.6 mmol) and 18-crown-6 (7.59 g, 28.7 mmol) in dry THF (100 mL) at 0°C under argon. Compound **13** (3.87 g, 15.9 mmol) was added at -78°C. The reaction mixture was stirred at room temperature for 3 h, neutralized with ammonium chloride, and then extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered, and concentrated. Methanol (10 mL) and 4 M hydrochloric acid (6 mL) were added to the residue, and the mixture was refluxed at 85°C for 30 min, neutralized

with sodium bicarbonate, and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/4) to give **32** (3.47 g, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65-7.61 (3 H, m), 7.23 (1 H, dd, *J* = 2.8, 8.0 Hz), 6.46 (1 H, d, *J* = 9.6 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.0, 153.0, 142.2, 134.7, 130.2, 120.4, 118.7, 118.0, 117.1.

4.2.3. Synthesis of 2-acetoxy-4-(diethylamino)benzaldehyde (16)

Acetic anhydride (7.0 mL) was added to a suspension of **15** (2.54 g, 13.2 mmol) in dry pyridine (10 mL) and dry methylene chloride (30 mL) at room temperature. After 30 min, the solvents were removed in vacuo to afford **16** (3.30 g, quant.) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.74 (1 H, s), 7.65 (1 H, d, *J* = 8.8 Hz), 6.55 (1 H, dd, *J* = 2.4, 8.8 Hz), 6.26 (1 H, d, *J* = 2.4 Hz), 3.41 (4 H, q, *J* = 7.2 Hz), 2.38 (3 H, s), 1.21 (6 H, t, *J* = 6.8 Hz).

4.2.4. Synthesis of 2-acetoxy-5-bromo-4-(diethylamino)benzaldehyde (17)

Bromine (2.87 g, 18.0 mmol) in acetic acid (15 mL) was added to a suspension of **16** (3.17 g, 13.5 mmol) in acetic acid (5 mL) at 0°C. The reaction mixture was stirred at room temperature for 4 h. The reaction was quenched with water at 0°C, and the mixture was neutralized with sodium bicarbonate, and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/5) to give **16** (2.05 g, 48%) as a brown oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.86 (1 H, s), 8.00 (1 H, s), 6.70 (1 H, s), 3.28 (4 H, q, *J* = 7.6 Hz), 2.38 (3 H, s), 1.14 (6 H, t, *J* = 6.9 Hz) ; ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.4, 153.8, 111.2, 109.6, 104.5, 99.5, 96.2, 95.9, 44.2, 12.5, 12.2.

4.2.5. Synthesis of 5-bromo-6-(diethylamino)coumarin (18)

Potassium bis(trimethylsylyl)amide (0.5 M solution in toluene, 17 mL, 8.50 mmol) was added to a solution of bis(2,2,2-trifluoromethyl)(methoxycarbonylmethyl)phosphonate (0.90 mL) and 18-crown-6 (2.96 g, 11.2 mmol) in dry THF (66 mL) at 0°C under argon. Compound **17** (1.32 g, 4.21 mmol) was added to the mixture at −78°C. The reaction mixture was stirred at room temperature for 1 d, and then neutralized with ammonium chloride, and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/5) to give **18** (601 mg, 48%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (1 H, s), 7.56 (1 H, d, *J* = 8.8 Hz), 6.94 (1 H, s), 6.28 (1 H, d, *J* = 9.6 Hz), 3.22 (4 H, q, *J* = 7.2 Hz), 1.10 (6 H, t, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.9, 154.1, 152.9, 142.3, 132.4, 115.2, 114.7, 114.6, 110.6, 46.4, 12.2.

4.2.6. Synthesis of 5-bromo-6-hydroxy-4-methylcoumarin (20)

A mixture of 4-bromoresorcinol (**19**, 2.29 g, 12.1 mmol), 3-oxobutanoic acid (1.6 mL), and conc. H_2SO_4 (15 mL) was stirred at 0°C to room temperature for 1 d. The reaction mixture was poured into ice water, and the precipitates were collected and washed with water to give **20** (2.23 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (1 H, s), 7.00 (1 H, s), 6.18 (1 H, s), 2.39 (3 H, s).

4.2.7. Synthesis of 5-bromo-6-methoxy-4-methylcoumarin (21)

A mixture of **41** (763 mg, 2.99 mmol), K_2CO_3 (516 mg, 3.73 mmol) in DMF (8.0 mL) was stirred for 15 min. Iodoethane (0.25 mL, 4.02 mmol) was added, and the reaction mixture was stirred for 1 d at room temperature. The mixture was quenched with 2 M hydrochloric acid, and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated to give **21** (809 mg, quant). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (1 H, s), 6.84 (1 H, s), 6.17 (1 H, s), 3.96 (3 H, s), 2.39 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.8, 158.5, 154.4, 151.7, 128.7, 114.6, 112.9, 107.5, 100.4, 56.9, 18.8.

4.2.8. Typical procedure for compounds 22 – 24 and 8a – f: Synthesis of 5-(*N*-*tert*-butoxycarbonyl-2-pyrrolyl)-6-(diethylamino)coumarin (23)

18 (138.5 mg, 0.47 mmol) was added to a solution of boronic acid (304.5 mg, 1.44 mmol), cesium fluoride (303 mg, 1.99 mmol), and $PdCl_2$(dppf) (81.2 mg, 0.10 mmol) in dry DMF (5 mL) at room temperature under argon. The reaction mixture was heated at 60°C for 3 h, then neutralized with ammonium chloride, and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/4) to give **23** (129 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (1 H, d, *J* = 9.2 Hz), 7.32 (1 H, m), 7.24 (1 H, s), 6.82 (1 H, s), 6.25 (1 H, t, *J* = 3.2 Hz), 6.19 (1 H, d, *J* = 9.2 Hz), 6.18 (1 H, m), 2.97 (4 H, br), 1.36 (9 H, s), 0.95 (6 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.9, 155.2, 154.4, 148.9, 143.5, 132.3, 130.5, 123.9, 122.1, 113.9, 112.3, 111.5, 111.0, 106.2, 83.5, 44.3, 27.8, 12.2.

Compounds **22** and **24** were synthesized similarly from compounds **14** and **21**, respectively.

22: 71%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (1 H, d, *J* = 9.6 Hz), 7.52 (1 H, dd, *J* = 2.3, 8.7 Hz), 7.47 (1 H, d, *J* = 2.3 Hz), 7.36 (1 H, m), 7.32 (1 H, d, *J* = 8.3 Hz), 6.45 (1 H, d, *J* = 9.2 Hz), 6.25 (1 H, t, *J* = 3.2 Hz), 6.22 (1 H, m), 1.40 (9 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.8, 153.2, 149.1, 143.4, 133.2, 133.0, 130.9, 128.0, 122.9, 118.1, 116.9, 116.1, 115.1, 110.8, 84.0, 27.2.

24: 91%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1 H, s), 7.37 (1 H, m), 6.81 (1 H, s), 6.27(1 H, t, *J* = 3.2 Hz), 6.19 (1 H, m), 6.16 (1 H, d, *J* = 1.4 Hz), 3.83 (3 H, s), 2.40 (3 H, d, *J* = 0.9 Hz), 1.40 (9 H, s)

Compounds **8a - e** were synthesized similarly from compounds **14**.

8a: 99%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (1 H, d, *J* = 9.6 Hz), 7.62 (1 H, dd, *J* = 2.3, 8.7 Hz), 7.54 (1 H, d, *J* = 2.3 Hz), 7.38 (1 H, d, *J* = 8.7 Hz), 7.25 (1 H, d, *J* = 5.0 Hz), 6.95 (1 H, d, *J* = 5.0 Hz), 6.47 (1 H, d, *J* = 9.6 Hz), 2.32 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.8, 153.2, 143.4, 135.8, 134.0, 132.7, 131.5, 131.3, 128.0, 124.1, 119.0, 117.3, 117.2, 15.0; HRMS (ESI+) Calcd. for $C_{14}H_{11}O_2S$ $[M + H]^+$: 243.0474. Found 243.0470.

8b: 44%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (1 H, dd, *J* = 2.0, 8.4 Hz), 7.78 (1 H, d, *J* = 2.0 Hz), 7.75 (1 H, d, *J* = 9.6 Hz), 7.39 (1 H, d, *J* = 8.8 Hz), 7.31 (1 H, d, *J* = 5.6 Hz), 7.02 (1 H, d, *J* = 5.6 Hz), 6.48 (1 H, d, *J* = 9.6 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.3, 153.6, 143.5, 134.2, 132.4, 130.4, 129.6, 128.8, 127.8, 124.4, 118.9, 118.0, 117.4; HRMS (ESI+) Calcd. for $C_{13}H_7ClNaO_2S$ $[M + Na]^+$: 284.9747. Found 284.9751.

8c: 23%; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.72 (1 H, d, *J* = 9.6 Hz), 7.70 (1 H, dd, *J* = 1.8, 8.5 Hz), 7.60 (1 H, d, *J* = 1.8 Hz), 7.32 (1 H, d, *J* = 8.7 Hz), 7.11 (1 H, d, *J* = 3.7 Hz), 6.75 (1 H, m), 6.45 (1 H, d, *J* = 9.6 Hz), 2.52 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.8, 153.1, 143.5, 140.5, 139.9, 131.6, 129.2, 126.6, 124.2, 123.6, 119.2, 117.5, 117.3, 15.6; HRMS (ESI+) Calcd. for $C_{14}H_{11}NaO_2S$ $[M + Na]^+$: 265.0294. Found 265.0293.

8d: 27%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (1 H, d, *J* = 9.6 Hz), 7.75 (1 H, dd, *J* = 2.3, 8.0 Hz), 7.69 (1 H, d, *J* = 1.8 Hz), 7.62 (1 H, d, *J* = 3.7 Hz), 7.41 (1 H, d, *J* = 8.7 Hz), 7.29 (1 H, d, *J* = 4.1 Hz), 6.51 (1 H, d, *J* = 9.6 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 154.6, 149.7, 142.9, 138.7, 129.9, 129.0, 125.6, 124.0, 119.5, 118.2, 118.1, 114.2, 109.1; HRMS (ESI+) Calcd. for $C_{14}H_8NO_2S$ $[M + H]^+$: 254.0270. Found 254.0274.

8e: 33%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (1 H, dd, *J* = 2.3, 8.5 Hz), 7.74 (1 H, d, *J* = 9.6Hz), 7.74 (1 H, d, *J* = 2.3 Hz), 7.68 (1 H, d, *J* = 3.7 Hz), 7.39 (1 H, d, *J* = 8.2 Hz), 7.33 (1 H, d, *J* = 4.1 Hz), 6.50 (1 H, d, *J* = 9.6 Hz), 2.58 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 190.4, 160.1, 154.3, 150.4, 143.8, 142.9, 133.4, 130.0, 129.5, 125.3, 124.3, 119.3, 117.8, 26.6; HRMS (ESI+) Calcd. for $C_{15}H_{11}O_3S$ $[M + H]^+$: 271.0423. Found 271.0426.

4.2.9. Typical procedure for compounds 25 – 27: Synthesis of 5-(*N*-*tert*-butoxycarbonyl-5-cyano-2-pyrrolyl)-6-(diethylamino)coumarin (26)

Chlorosulfonyl isocyanate (0.3 mL) was added to a solution of **23** (376 mg, 0.98 mmol) in dry THF (20 mL) at -78°C. After 1 h, DMF (0.5 mL) was added. The reaction mixture was allowed to warm

to room temperature, then poured into water, and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/3) to give **26** (309 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (1 H, d, *J* = 9.2 Hz), 7.25 (1 H, s), 7.02 (1 H, d, *J* = 3.6 Hz), 6.87 (1 H, s), 6.27 (1 H, d, *J* = 3.6 Hz), 6.24 (1 H, d, *J* = 9.2 Hz), 2.95 (4 H, br), 1.49 (9 H, s), 0.97 (6 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.3, 155.6, 154.1, 146.8, 143.1, 138.1, 130.2, 124.7, 122.3, 113.5, 113.3, 112.2, 107.2, 105.1, 87.1, 44.5, 27.7, 11.9.

Compounds **25** and **27** were synthesized similarly from compounds **22** and **24**, respectively.
25: 90%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (1 H, d, *J* = 9.6 Hz), 7.49 (1 H, dd, *J* = 1.8, 8.6 Hz), 7.48 (1 H, d, *J* = 1.8 Hz), 7.37 (1 H, d, *J* = 8.7 Hz), 7.01 (1 H, m), 6.48 (1 H, d, *J* = 9.6 Hz), 6.29 (1 H,m), 1.50 (9H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.5, 154.0, 147.2, 143.1, 138.5, 132.8, 128.9, 128.2, 124.6, 118.5, 117.6, 116.8, 114.7, 113.3, 106.1, 87.7, 27.7.
27: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1 H, s), 7.00 (1 H, d, *J* = 3.7 Hz), 6.84 (1 H, s), 6.24 (1 H, d, *J* = 3.7 Hz), 6.19 (1 H, d, *J* = 0.9 Hz), 3.84 (3 H, s), 2.41 (3 H, d, *J* = 0.9 Hz), 1.51 (9 H, s).

4.2.10. Typical procedure for compounds 7a and 10a: Synthesis of 5-(5-cyano-2-pyrrolyl)coumarin (7a)

A solution of **25** (888 mg, 2.64 mmol) in water (50 mL) was heated at 100°C for 3 h. The reaction mixture was extracted with AcOEt. The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated to give **7a** (621mg, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.26 (1 H, br), 7.74 (1 H, d, *J* = 9.6 Hz), 7.68 (1 H, dd, *J* = 2.4, 8.6 Hz), 7.62 (1 H, d, *J* = 2.4 Hz), 7.40 (1 H, d, *J* = 8.8 Hz), 6.94 (1 H, m), 6.55 (1 H, m), 6.50 (1 H, d, *J* = 9.6 Hz); ^{13}C NMR (150 MHz, DMSO-d_6) δ 159.7, 152.8, 144.0, 135.6, 128.3, 127.3, 124.3, 121.0, 119.1, 117.0, 116.9, 114.8, 107.7, 100.9; HRMS (ESI+) Calcd. for $C_{14}H_9N_2O_2$ $[M + H]^+$: 237.0659. Found 237.0658.

Compounds **10a** was synthesized similarly from compound **27**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (1 H, s), 6.96 (1 H, s), 6.92 (1 H, m), 6.63 (1 H, m), 6.22 (1 H, d, *J* = 1.2 Hz), 4.10 (3 H, s), 2.46 (3 H, d, *J* = 1.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.0, 158.8, 154.0, 153.6, 132.6, 123.9, 120.4, 116.6, 115.2, 113.1, 111.8, 110.7, 100.2, 100.1, 56.6, 18.3; HRMS (ESI+) Calcd. for $C_{16}H_{13}N_2O_3$ $[M + H]^+$: 281.0921. Found 281.0922.

4.2.11. Synthesis of 5-(5-cyano-2-pyrrolyl)-6-(diethylamino)coumarin (9a)

A solution of **26** (61.9 mg, 0.20 mmol) in 8% hydrochloric acid/ethanol (3 mL) was heated at 60°C for 3 h. The reaction mixture was extracted with methylene chloride. The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated. The residue was purified by column

chromatography (silica gel, ether) to give **9a** (58.7 mg, 95%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.0 (1 H, br), 7.70 (1 H, s), 7.69 (1 H, d, *J* = 10.1 Hz), 7.19 (1 H, s), 6.89 (1 H, d, *J* = 2.3 Hz), 6.55 (1 H, d, *J* = 2.8 Hz), 6.40 (1 H, d, *J* = 9.6 Hz), 3.10 (4 H, q, *J* = 6.9 Hz), 1.07 (6 H, t, *J* = 6.9 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.5, 153.8, 150.8, 142.9, 135.4, 127.0, 123.8, 120.7, 116.3, 116.2, 114.9, 111.9, 107.8, 100.2, 48.4, 12.0; HRMS (ESI+) Calcd. for $C_{18}H_{18}N_3O_2$ $[M + H]^+$: 308.1394. Found 308.1392.

4.2.12. Typical procedure for *N*-alkylation of pyrrole ring: Synthesis of 5-(5-cyano-1-methyl-2-pyrrolyl)-6-(diethylamino)coumarin (9b)

Sodium hydride (25.2 mg, 0.63 mmol) was washed with *n*-hexane. A solution of **9a** (46.1 mg, 0.15 mmol) in dry DMF (1.5 mL) was added to a suspension of sodium hydride in dry DMF (1 mL) at room temperature. After 30 min, iodoethane (65.4 mg, 0.46 mmol) was added. The reaction mixture was stirred for 2 h at room temperature, then poured into water, and extracted with ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, filtered, and concentrated to give **9b** (44.1 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (1 H, d, *J* = 9.2 Hz), 7.26 (1 H, s), 6.89 (1 H, s), 6.88 (1 H, d, *J* = 4.0 Hz), 6.25 (1 H, d, *J* = 9.2 Hz), 6.18 (1 H, d, *J* = 3.6 Hz), 3.52 (3 H, s), 3.00 (4 H, q, *J* = 7.6 Hz), 0.95 (6 H, t, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.0, 155.7, 153.4, 142.7, 138.3, 132.5, 120.4, 119.8, 114.1, 113.3, 112.0, 109.6, 106.6, 104.7, 45.6, 33.4, 12.2; HRMS (ESI+) Calcd. for $C_{19}H_{20}N_3O_2$ $[M + H]^+$: 322.1550. Found 320.1556.

Compounds **7b-l**, **9c-e**, and **10b-h** were synthesized similarly.

7b: 95%; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.73 (1 H, d, *J* = 9.6 Hz), 7.53 (1 H, dd, *J* = 2.4, 8.8 Hz), 7.49 (1 H, d, *J* = 2.0 Hz), 7.43 (1 H, d, *J* = 8.8 Hz,), 6.88 (1 H, d, *J* = 3.6 Hz), 6.51 (1 H, d, *J* = 9.6 Hz), 6.21 (1 H, d, *J* = 4.0 Hz), 4.11 (2 H, q, *J* = 7.2 Hz), 1.35 (3 H, t, *J* = 7.6 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 154.0, 142.8, 137.4, 132.3, 128.3, 128.0, 120.0, 119.1, 117.7, 117.5, 113.9, 110.5, 104.8, 41.5, 16.7; HRMS (ESI+) Calcd. for $C_{16}H_{13}N_2O_2$ $[M + H]^+$: 265.0972. Found 265.0976.

7c: 92%; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.72 (1 H, d, *J* = 9.6 Hz), 7.52 (1 H, dd, *J* = 2.4, 8.0 Hz), 7.48 (1 H, d, *J* = 2.0 Hz), 7.42 (1 H, d, *J* = 8.8 Hz), 6.87 (1 H, d, *J* = 4.0 Hz), 6.51 (1 H, d, *J* = 9.6 Hz), 6.21 (1 H, d, *J* = 4.4 Hz), 4.06 (t, *J* = 7.6 Hz, 2H), 1.66 (2 H, quint, *J* = 7.2 Hz), 1.23-1.13 (6 H, m), 0.81 (3 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 153.9, 142.8, 137.6, 132.0, 128.3, 119.9, 119.1, 117.7, 117.4, 114.0, 110.6, 105.1, 46.6, 31.1, 31.0, 26.0, 22.3, 13.8; HRMS (ESI+) Calcd. for $C_{20}H_{21}N_2O_2$ $[M + H]^+$: 321.1598. Found 321.1602.

7d: 99%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (1 H, d, *J* = 9.6 Hz), 7.51 (1 H, dd, *J* = 2.4, 8.0 Hz),

7.47 (1 H, d, *J* = 1.6 Hz), 7.42 (1 H, d, *J* = 8.8 Hz), 6.86 (1 H, d, *J* = 4.0 Hz), 6.51 (1 H, d, *J* = 9.6 Hz), 6.20 (1 H, d, *J* = 4.0 Hz), 3.94 (2 H, d, *J* = 8.0 Hz), 1.63-1.50 (4 H, m), 1.38-1.30 (2 H, m), 1.13-1.00 (3 H, m), 0.78-0.68 (2 H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.2, 153.8, 142.9, 138.1, 132.5, 128.4, 119.8, 119.0, 117.7, 117.4, 114.2, 110.7, 105.7, 52.7, 39.3, 30.2, 25.9, 25.4; HRMS (ESI+) Calcd. for $C_{21}H_{21}N_2O_2$ $[M + H]^+$: 333.1598. Found 333.1593.

7e: 12%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (1 H, d, *J* = 9.6 Hz), 7.59 (1 H, s), 7.58 (1 H, dd, *J* = 2.4, 5.6 Hz), 7.49 (1 H, d, *J* = 9.2 Hz), 7.00 (1 H, d, *J* = 4.0 Hz), 6.55 (1 H, d, *J* = 9.6 Hz), 6.37 (1 H, d, *J* = 4.0 Hz), 4.89 (2 H, s); ^{13}C NMR (150 MHz, DMSO-d_6) δ 153.7, 143.9, 138.6, 132.2, 129.0, 125.7, 122.0, 120.5, 119.2, 117.2, 115.3, 112.8, 111.6, 105.0, 90.7, 34.4; HRMS (ESI+) Calcd. for $C_{16}H_{10}N_3O_2$ $[M + H]^+$: 276.0768. Found 276.0767.

7f: 45%; ^{1}H NMR (400MHz, $CDCl_3$) δ 7.56 (1 H, d, *J* = 9.6 Hz), 7.42 (1 H, dd, *J* = 2.0, 8.8 Hz), 7.32(1 H, d, *J* = 2.0 Hz), 7.32 (1 H, d, *J* = 8.8 Hz), 7.29 (3 H, m), 6.95 (1 H, d, *J* = 4.0 Hz), 6.92 (2 H, m), 6.45 (1 H, d, *J* = 9.6 Hz), 6.32 (1 H, d, *J* = 4.0 Hz), 5.27 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 154.0, 142.8, 138.3, 136.5, 132.4, 129.0, 128.4, 128.1, 127.7, 126.1, 120.3, 118.9, 117.6, 117.4, 113.9, 110.9, 106.1, 49.9; HRMS (ESI+) Calcd. for $C_{21}H_{15}N_2O_2$ $[M + H]^+$: 327.1128. Found 327.1127.

7g: 44%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (2 H, d, *J* = 8.8 Hz), 7.60 (1 H, d, *J* = 9.6 Hz), 7.39 (1 H, dd, *J* = 2.0, 8.4 Hz), 7.37 (1 H, d, *J* = 8.8 Hz), 7.34 (1 H, d, *J* = 2.0 Hz), 7.01 (2 H, d, *J* = 8.0 Hz), 7.00 (1 H, d, *J* = 4.0 Hz), 6.48 (1 H, d, *J* = 9.6 Hz), 6.36 (1 H, d, *J* = 4.0 Hz), 5.33 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.8, 154.1, 142.5, 141.5, 138.4, 132.8, 132.1, 128.3, 127.2, 126.7, 120.8, 119.1, 117.9, 117.6, 113.4, 112.2, 111.4, 106.0, 49.4; HRMS (ESI+) Calcd. for $C_{22}H_{14}N_3O_2$ $[M + H]^+$: 352.1081. Found 352.1079.

7h: 59%; ^{1}H NMR (400 MHz, $CDCl_3$) δ8.17 (2 H, d, *J* = 8.8 HzH), 7.61 (1 H, d, *J* = 9.6 Hz), 7.40 (1 H, dd, *J* = 2.0, 8.4 Hz), 7.37 (1 H, d, *J* = 2.0 Hz), 7.35 (1 H, d, *J* = 8.4 Hz), 7.07 (2 H, d, *J* = 9.2 Hz), 7.01 (1 H, d, *J* = 4.4 Hz), 6.48 (1 H, d, *J* = 9.6 Hz), 6.38 (1 H, d, *J* = 3.6 Hz), 5.38 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.9, 154.1, 147.6, 143.5, 142.5, 138.4, 132.1, 128.3, 127.4, 126.9, 124.3, 120.9, 119.2, 118.0, 117.7, 113.3, 111.5, 106.0, 49.2; HRMS (ESI+) Calcd. for $C_{21}H_{14}N_3O_4$ $[M + H]^+$: 372.0979. Found 372.0976.

7i: 49%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (1 H, d, *J* = 9.6 Hz), 7.42 (1 H, dd, *J* = 2.0, 8.8 Hz), 7.35 (1 H, d, *J* = 8.4 Hz), 7.34 (1 H, d, *J* = 2.0 Hz), 6.99 (2 H, t, *J* = 8.8 Hz), 6.96 (1 H, d, *J* = 4.0 Hz), 6.89 (2 H, dd, *J* = 5.6, 8.8 Hz), 6.48 (1 H, d, *J* = 9.6 Hz), 6.31 (1 H, d, *J* = 4.0 Hz), 5.24 (2 H, s);

^{13}C NMR (150 MHz, $CDCl_3$) δ 163.5, 161.1, 160.0, 154.0, 142.7, 138.2, 132.2, 128.4, 128.0, 120.4, 119.0, 117.7, 117.4, 116.1, 115.9, 113.7, 111.1, 105.9, 49.2; HRMS (ESI+) Calcd. for $C_{21}H_{14}FN_2O_2$ $[M + H]^+$: 345.1034. Found 345.1039.

7j: 37%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (1 H, d, *J* = 9.6 Hz), 7.41 (1 H, dd, *J* = 1.6, 8.4 Hz), 7.37 (1 H, d, *J* = 1.6 Hz), 7.36 (1 H, d, *J* = 8.0 Hz), 7.00 (1 H, d, *J* = 4.4 Hz), 6.74 (1 H, tt, *J* = 2.4, 8.8 Hz), 6.48 (1 H, d, *J* = 9.6 Hz), 6.43 (2 H, br d, *J* = 6.0 Hz), 6.36 (1 H, d, *J* = 4.0 Hz), 5.25 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.6, 162.1, 159.9, 154.1, 142.6, 140.4, 138.3, 132.2, 128.3, 127.2, 120.8, 119.1, 117.9, 117.6, 113.4, 111.3, 109.1, 106.0, 103.8, 49.0; HRMS (ESI+) Calcd. for $C_{21}H_{12}F_2N_2NaO_2$ $[M + Na]^+$: 385.0759. Found 385.0760.

7k: 51%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (1 H, d, *J* = 9.6 Hz), 7.46 (1 H, dd, *J* = 1.6, 6.4 Hz), 7.45 (1 H, d, *J* = 1.6 Hz), 7.41 (1 H, d, *J* = 9.2 Hz), 6.93 (1 H, d, *J* = 4.4 Hz), 6.52 (1 H, d, *J* = 9.6 Hz), 6.25 (1 H, d, *J* = 4.0 Hz), 5.35 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.9, 154.2, 142.5, 138.6, 132.6, 128.7, 127.2, 121.1, 119.1, 118.0, 117.6, 117.6, 113.0, 111.2, 105.7, 38.6; HRMS (ESI+) Calcd. for $C_{21}H_{10}F_5N_2O_2$ $[M + H]^+$: 417.0657. Found 417.0650.

7l: 27%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83-7.79 (2 H, m), 7.71-7.69 (1 H, m), 7.56-7.47 (3 H, m), 7.44 (1 H, dd, *J* = 2.4, 8.8 Hz), 7.31-7.26 (3 H, m), 7.09 (1 H, dd, *J* = 2.0, 8.0 Hz), 7.00 (1 H, d, *J* = 4.0 Hz), 6.41 (1 H, d, *J* = 9.6 Hz), 6.35 (1 H, d, *J* = 4.0 Hz), 5.34 (2 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 153.9, 142.7, 138.3, 134.0, 133.2, 132.8, 132.4, 129.0, 128.4, 127.8, 127.7, 127.7, 126.6, 126.4, 125.1, 123.7, 120.3, 118.9, 117.5, 117.3, 113.8, 111.0, 106.1, 50.1; HRMS (ESI+) Calcd. for $C_{25}H_{17}N_2O_2$ $[M + H]^+$: 377.1285. Found 377.1274.

9c: 99%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (1 H, d, *J* = 9.2 Hz), 7.26 (1 H, s), 6.90 (1 H, d, *J* = 4.0 Hz), 6.88 (1 H, s), 6.25 (1 H, d, *J* = 8.8 Hz), 6.14 (1 H, d, *J* = 4.0 Hz), 3.95 (2 H, q, *J* = 7.2 Hz), 3.01 (4 H, q, *J* = 7.2 Hz), 1.17 (3 H, t, *J* = 7.6 Hz), 0.95 (6 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.1, 154.0, 142.8, 137.4, 132.3, 128.3, 128.0, 120.0, 119.0, 117.7, 117.5, 113.9, 110.6, 104.8, 41.5, 16.7; HRMS (ESI+) Calcd. for $C_{20}H_{22}N_3O_2$ $[M + H]^+$: 336.1707. Found 336.1706.

9d: 37%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (1 H, d, *J* = 9.2 Hz), 7.35 (1 H, s), 7.01 (1 H, d, *J* = 4.0 Hz), 6.97 (1 H, s), 6.30 (1 H, d, *J* = 9.2 Hz), 6.30 (1 H, d, *J* = 4.0 Hz), 4.54 (2 H, s), 3.03 (4 H, q, *J* = 6.8 Hz), 0.99 (6 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.6, 156.1, 152.5, 142.6, 138.5, 133.0, 122.0, 119.0, 114.4, 113.0, 112.4, 111.9, 111.1, 108.0, 105.2, 46.2, 34.3, 12.0; HRMS (ESI+) Calcd. for $C_{20}H_{19}N_4O_2$ $[M + H]^+$: 347.1503. Found 347.1494.

9e: 36%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (1 H, d, *J* = 9.2 Hz), 7.14 (3 H, m), 7.02 (1 H, s), 6.94 (1 H, d, *J* = 4.0 Hz), 6.89 (1 H, s), 6.71 (2 H, m), 6.20 (1 H, d, *J* = 9.6 Hz), 6.20 (1 H, d, *J* = 3.6 Hz), 5.12 (2 H, s), 3.02 (4 H, q, *J* = 7.2 Hz), 0.99 (6 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.2, 155.7, 153.2, 142.9, 138.2, 136.5, 133.1, 128.7, 127.9, 127.1, 120.9, 120.7, 114.4, 113.3, 112.13, 110.4, 106.7, 104.8, 50.6, 45.9, 12.4; HRMS (ESI+) Calcd. for $C_{25}H_{24}N_3O_2$ $[M + H]^+$: 398.1863. Found 398.1869.

10b: 66%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (1 H, s), 6.92 (1 H, s), 6.88 (1 H, dd, *J* = 1.4, 4.0 Hz), 6.21 (1 H, s), 6.17 (1 H, dd, *J* = 1.4, 4.0 Hz), 3.91 (3 H, s), 3.57 (3 H, s), 2.40 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.7, 160.2, 155.9, 152.1, 135.3, 128.0, 119.2, 117.4, 114.1, 113.5, 112.8, 110.7, 105.5, 99.6, 56.2, 33.5, 18.7; HRMS (ESI+) Calcd. for $C_{17}H_{15}N_2O_3$ $[M + H]^+$: 295.1077. Found 295.1077.

10c: 29%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (1 H, s), 7.21 (1 H, s), 7.05 (1 H, d, *J* = 3.6 Hz), 6.28 (1 H, d, *J* = 1.2 Hz), 6.22 (1 H, d, *J* = 3.6 Hz,), 3.87 (3 H, s), 3.86 (2 H, q, *J* = 7.2 Hz), 2.40 (3 H, d, *J* = 0.8 Hz), 1.18 (3 H, t, *J* = 6.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.6, 160.2, 155.9, 152.1, 134.2, 128.1, 119.8, 117.5, 114.1, 113.5, 112.8, 110.9, 103.9, 99.6, 56.2, 41.9, 18.7, 16.3; HRMS (ESI+) Calcd. for $C_{18}H_{17}N_2O_3$ $[M + H]^+$: 309.1234. Found 309.1231.

10d: 40%; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.43 (1 H, s), 6.91 (1 H, s), 6.88(1 H, d, *J* = 4.1 Hz), 6.21(1 H, s), 6.13 (1 H, d, *J* = 4.1 Hz), 3.89-.386 (5 H, m), 2.40 (3 H, s), 1.61 (2 H, t, *J* = 7.3 Hz), 1.18-1.09 (6 H, m), 0.77 (3 H, t, *J* = 6.9 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.9, 160.3, 155.9, 152.3, 134.6, 128.2, 119.8, 117.8, 114.4, 113.6, 112.9, 111.0, 104.6, 99.7, 56.3, 47.1, 31.2, 31.0, 26.2, 22.6, 18.9, 14.0; HRMS (ESI+) Calcd. for $C_{22}H_{25}N_2O_3$ $[M + H]^+$: 365.1860. Found 365.1850.

10e: 38%; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40(1 H, s), 6.89(1 H, s), 6.86(1 H, d, *J* = 4.0 Hz), 6.20(1 H, s), 6.10(1 H, d, *J* = 3.6 Hz), 3.86(3 H, s), 3.71(2 H, d, *J* = 7.6 Hz), 2.38(3 H, s), 1.55-1.59(4 H, m), 1.35-1.38(2 H, m), 0.99-1.09(3 H, m), 0.66-0.75(2 H, m); ^{13}C NMR (150 MHz, DMSO-d_6) δ 159.9, 159.8, 155.2, 153.3, 135.1, 128.4, 119.5, 116.9, 114.3, 112.9, 111.8, 111.3, 103.8, 99.9, 56.5, 52.3, 29.7, 25.5, 25.0, 18.2; HRMS (ESI+) Calcd. for $C_{23}H_{25}N_2O_3$ $[M + H]^+$: 377.1860. Found 377.1865.

10f: 33%; ^{1}H NMR (400 MHz, $CDCl_3$) δ7.23-7.20 (3 H, m), 7.18 (1 H, s), 6.96(1 H, d, *J* = 4.0 Hz), 6.87-6.85 (2 H, m), 6.85 (1 H, s), 6.23 (1 H, d, *J* = 3.6 Hz), 6.14 (1 H, d, *J* = 1.6 Hz), 5.09 (2 H, s), 3.77 (3 H, s), 2.16 (3 H, d, *J* = 1.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.6, 160.2, 155.8, 152.1, 136.6, 134.8, 128.6, 128.2, 127.8, 126.5, 119.9, 117.2, 114.0, 113.2, 112.6, 111.5, 105.3, 99.4, 56.1, 50.3, 18.4; HRMS (ESI+) Calcd. for $C_{23}H_{19}N_2O_3$ $[M + H]^+$: 371.1390. Found 371.1400.

10g: 22%; ^{1}H NMR (400 MHz, CD_3OD) δ 7.62 (2 H, d, *J* = 8.4 Hz), 7.45 (1 H, s), 7.06(2 H, d, *J* = 8.4 Hz), 7.04 (1 H, d, *J* = 4.0 Hz), 7.02 (1 H, s), 6.32 (1 H, d, *J* = 4.0 Hz), 6.18 (1 H, d, *J* = 0.8 Hz), 5.25 (2 H, s), 3.76 (3 H, s), 2.31 (3 H, d, *J* = 0.8 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.6, 160.1, 156.1, 151.9, 141.9, 135.3, 132.6, 128.3, 127.3, 120.7, 118.4, 116.8, 113.8, 113.6, 113.1, 112.0, 111.9, 105.4, 99.8, 56.2, 49.9, 18.7; HRMS (ESI+) Calcd. for $C_{24}H_{18}N_3O_3$ $[M + H]^+$: 396.1343. Found 396.1332.

10h: 44%; ^{1}H NMR (400 MHz, $CDCl_3$) δ6.95-6.87 (7 H, m), 6.22-6.17 (2 H, m), 5.06(2 H, s), 3.78 (3 H, s), 2.23 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.1, 161.5, 160.7, 160.2, 155.9, 152.2, 134.9, 132.5, 132.4, 128.6, 128.5, 128.3, 120.3, 117.2, 115.8, 115.6, 114.1, 113.5, 112.9, 111.7, 105.2, 99.6, 56.3, 49.8, 18.6; HRMS (ESI+) Calcd. for $C_{23}H_{18}FN_2O_3$ $[M + H]^+$: 389.1296. Found 389.1291.

4.2.13. Synthesis of 5-(5-cyano-1-methyl-2-pyrrolyl)-6-hydroxycoumarin (11)

Boron tribromide (1 M solution in dichloromethane, 4 mL) was added to a solution of **10b** (48.6 mg, 0.17 mmol) in dichloromethane (6.0 mL) at –78°C under argon. The mixture was stirred at room temperature for 2 d, then poured into water, and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over magnesium sulfate, filtered, and concentrated. The residue was purified by column chromatography (silica gel, AcOEt / *n*-hexane = 1/2) to give **11** (21.1 mg, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.13 (1 H, s), 7.58 (1 H, d, *J* = 1.6 Hz), 7.03 (1 H, dd, *J* = 2.0, 4.4 Hz), 6.89 (1 H, d, *J* = 1.6 Hz), 6.27 (1 H, dd, *J* = 2.0, 4.0 Hz), 6.20 (1 H, s), 3.56 (3 H, s), 2.38 (3 H, s); ^{13}C NMR (150 MHz, $CDCl_3$) δ 160.0, 158.9, 154.9, 153.5, 136.1, 128.9, 119.3, 115.7, 114.2, 112.2, 111.0, 110.8, 103.8, 102.5, 33.5, 18.1; HRMS (ESI+) Calcd. for $C_{16}H_{12}N_2NaO_3$ $[M + Na]^+$: 303.0740. Found 303.0739.

4.3 Biology

4.3.1. Alkaline phosphatase assay

Alkaline phosphatase assay was performed as reported.[22] T47D breast-carcinoma cells were cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum. Cells were plated in 96-well plates at 10^4 cell/well and incubated overnight (37°C, 5% CO_2 in air). The next day, cells were treated with fresh medium containing test compound, and further incubated for 24 h. The medium was aspirated and the cells were fixed with 100 μL of 1.8% formalin (in PBS). The fixed cells were washed with PBS and 75 μL of assay buffer (1 mg/mL *p*-nitrophenol phosphate in diethanolamine water solution, pH 9.0, 2 mM $MgCl_2$) was added. The mixture was incubated at room temperature with shielding from light for 2 h, and then the reaction was terminated by the addition of 100 μL of 1 M NaOH. The absorbance at 405 nm was measured using a plate reader.

4.3.2. hPR-binding assay

PR-binding experiments were performed as reported, using recombinant hPR-LBD purchased from Invitrogen.[22] hPR-LBD was diluted with buffer (20 mM Tris.HCl, 300 mM NaCl, 1 mM EDTA, 5 mM DTT, pH 8.0) to 5 nM and 300 μL aliquots were incubated in the dark at 4°C with 4 nM [1,2,6,7-^{3}H]progesterone (Perkin Elmer) and reference or test compounds (dissolved in DMSO; final concentration of DMSO was 3%). Nonspecific binding was assessed by addition of a 200-fold excess of nonradioactive progesterone. After 24 h, 30 μL of Dextran T-70/c-globulin-coated charcoal suspension was added to the ligand/protein mixtures (1% activated charcoal, 0.05% γ-globulin, 0.05% Dextran 70, final concentrations) and incubated at 4°C for 5 min. The charcoal was removed by centrifugation for 5 min at 1300 g, and the radioactivity of the supernatant was measured in Ultima Gold scintillation cocktail (Perkin Elmer) by using a liquid scintillation counter. All experiments were performed in duplicate.

4.3.3. SC-3 Growth Inhibition Assay

SC-3 Growth inhibition assay was performed as reported.[22,26] SC-3 cells were cultured in MEMα (Wako Co.) supplemented with 2% FBS and 1 nM DHT at 37°C under 5% CO_2. All experiments were performed in triplicate or more. Cells were trypsinized and diluted to 20,000 cells/mL with MEMα supplemented with 2% charcoal-stripped FBS. This cell suspension was seeded in 96-well plates at a volume of 100 μL and incubated at 24 h. After removal of 10 μL of medium from each well, 10 μL of the drug solution, supplemented with serial dilutions of the test compounds or DMSO as a dilution control in the presence of 1 nM DHT, was added. Then the plates were incubated at 37°C under 5% CO_2 for 3 days, and the cell number was determined using a Cell Counting Kit-8 (Dojindo). A 10 μL aliquot of WST-8 was added to each well of the microcultures, and the cells were incubated for 2 h. The absorbance at 450 nm was measured with a microplate reader. This parameter is related to the number of living cells in the culture.

4.3.4. hGR-binding assay

hGR-binding experiments were performed at Caliper Life Science (US), according to the reported method.[22] The hGR was diluted with binding buffer (50 mM KH_2PO_4 pH 7.4 with 10 mM sodium molybdate and 1 mM ditiothreitol) to yield a final protein concentration of 1.25 nM in the assay tubes. The final incubation conditions were: [^{3}H]dexamethasone, 10^{-9} M; triamcinolone acetonide, 10^{-5} M (for non-specific binding determination only); test compounds, 10^{-7} to 10^{-5} M, 0.4% DMSO. After 18 h, the bound ligand was assayed by vacuum filtration onto glass fiber filters and radioactivity was counted in 50 μl of scintillation cocktail (Microscint-20). All experiments were performed in duplicate.

4.3.5. rMR-binding assay

rMR-binding experiments were performed at Caliper Life Science (US) , according to the reported method.[22] The rMR was diluted with binding buffer (20 mM HEPES pH 7.4, 15 mM sodium molybdate, 1 mM dithiothreitol, 10% glycerol and 1 mM EDTA). The concentration for the MR receptor tissue preparation was optimized for each tissue preparation, and was 16.7 mg wet weight/mL in the final reaction for the present assays. The final incubation conditions were: [^{3}H]aldosterone, 2×10^{-9} M; spironolactone, 10^{-6} M (for non-specific binding determination only); test compounds, 10^{-7} to 10^{-5} M; 0.4% DMSO. After 18-20 h, the unbound ligand was removed by absorption on dextran-coated charcoal, followed by centrifugation. The radioactivity of bound ligand in 200 µl of the supernatant was counted in 6 mL of scintillation cocktail (Luma Safe). All experiments were performed in duplicate.

4. 4. Fluorescence study

Fluorescence spectra were recorded with a JASCO FP-6600. Fluorescence quantum yields (Φ_{fl}) were determined using quinine sulfate (0.577) in 0.1 M H_2SO_4 as a standard. Fluorescence spectra in the presence of PR-LBD were measured in the same buffer as that used for hPR binding assay (50 mM KH_2PO_4 pH 7.4 with 10 mM sodium molybdate and 1 mM dithiothreitol).

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Graphical Abstract

Development of 6-Arylcoumarins as Nonsteroidal Progesterone Antagonists. Structure-activity Relationships and Fluorescence Properties

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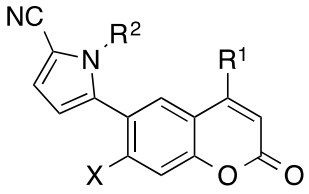

		IC_{50}
7c	R^1 = H, R^2 = *n*-hex, X = H	34 nM
9b	R^1 = H, R^2 = Me, X = NEt_2	240 nM
10b	R^1, R^2 = Me, X = OMe	24 nM