Synthesis and Anti-tumor Activities of Novel Oxazinyl Isoflavonoids

Dun Wang,*[,a] Like Hou,[a] Lirong Wu,[a] and Xin Yu[b]

[a] *College of Pharmacential Engineering, Shenyang Pharmaceutical University; Shenyang 110016, China: and* [b] *Medical & Regulatory Affairs Department, Fresenisu Kabi (China) Co., Ltd., BD; Beijing 100007, China.*

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The design, synthesis and biological evaluation of a novel series of oxazinyl isoflavonoids is described. Several analogs were shown to exhibit growth inhibitory effects against SKOV-3, DU-145 and HL-60 human colon cancer cell lines with IC_{50} values in the micromolar range. The cellular potency of compounds 7e and 12h were found to have greater *in vitro* inhibitory activities than phenoxodiol, the parental compound currently in late-stage clinical trials for the treatment of cancer. The results shown are suitable for further lead optimization.

Key words anti-tumor activity; oxazinyl isoflavonoid compound; synthesis

With the changes in the current living environment, cancer has become the second leading cause of death in developed countries. Over 1 million cases of cancer occur in the United States annually, and cancer-related deaths are estimated to reach 12 million worldwide by the year 2015.[1)] Therefore, a new generation of useful anticancer chemotherapy agents is becoming more urgent.

Isoflavonoids, which are present in vegetables, beans, peas, and other legumes, are one of the principal plant hormones known to possess the potential to regulate plant cycle kinetics and death and may have similar effects in animals. A number of naturally occurring plant hormones, including isoflavonoids and particularly daidzein and genistein, have been shown to exhibit biological activity in mammalian cells by regulating various functions, such as cell-cycle progression, mitotic arrest and apotosis.[2,3)] Specifically, genistein was well tolerated, with minimal toxicity, in normal tissues, and its effects were seen in multiple tumor types, including prostate, breast and pancreatic tumors. However, its limited bioavailability and extensive metabolism led to difficulties in attaining adequate plasma concentrations, resulting in limited utility and dissemination in the clinic.[4)] For phenoxodiol (2*H*-1-benzopyran-7-ol, 3-[4-hydrophenyl], Fig. 1), an analogue of genistein, a broad range of activities against human cancer cells was found, inducing mitotic arrest (G_1 phase of mitosis), terminal differentiation and apoptosis. Due to these findings, phenoxodiol was granted Fast Track status by the Food and Drug Administration (FDA) to be developed as a chemo-sensitizer for platinums and taxanes in the treatments of recurrent ovarian and hormone-refractory prostate cancers.[5)] Like genistein, phenoxodiol features a diphenolic ring structure, which may be essential for maintaining the free radical scavenging effect of the drug, while also being largely responsible for its ease of oxidation when standing for a long period.

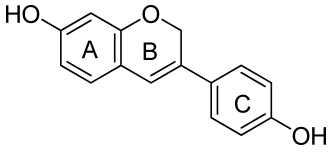


Fig. 1. The Structure of Phenoxodiol

In this paper, our goal was to generate highly potent, novel isoflavonoid derivatives that may have improved stabilities. We chose isoflav-3-ene (the structural skeleton of phenoxodiol), isoflavanone and isoflavane as the mother nuclei and made the following modifications: (1) the integration of oxazinyl with the A rings of the mother nuclei to protect the phenolic groups on the 7-positions; (2) the integration of oxazinyl with the C rings of the mother nuclei to protect the phenolic groups on the 4′-positions; (3) the introduction of various substituents on the N-positions of the oxazinyl rings in order to understand the effects of the different groups on the activity; and (4) the methylation of the phenolic groups on the 7- or 4′-positions of the mother nuclei to understand their effects on activity. Overall, thirty oxazinyl isoflavonoid compounds were synthesized, and their *in vitro* anti-tumor activities were evaluated.

Synthesis

In this paper, four types of oxazinyl isoflavonoid compounds were designed and synthesized (Fig. 2). The target compounds were obtained as described in Charts 1–5, and the substituents of compounds **7a**–**18d** are listed in Table 1.

Following an established method,[6)] intermediates **4a**–**c** were synthesized by reacting resorcinol with substituted phenylacetic acid in boron trifluoride etherate ($BF_3/(CH_3)_2O$) at 80°C for 4 h and then treating with *N*,*N*-dimethylformamide (DMF) and methanesulfonyl chloride. Subsequently, treatment of

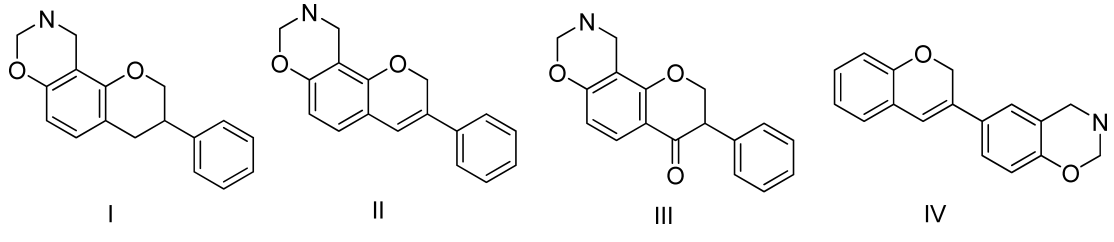
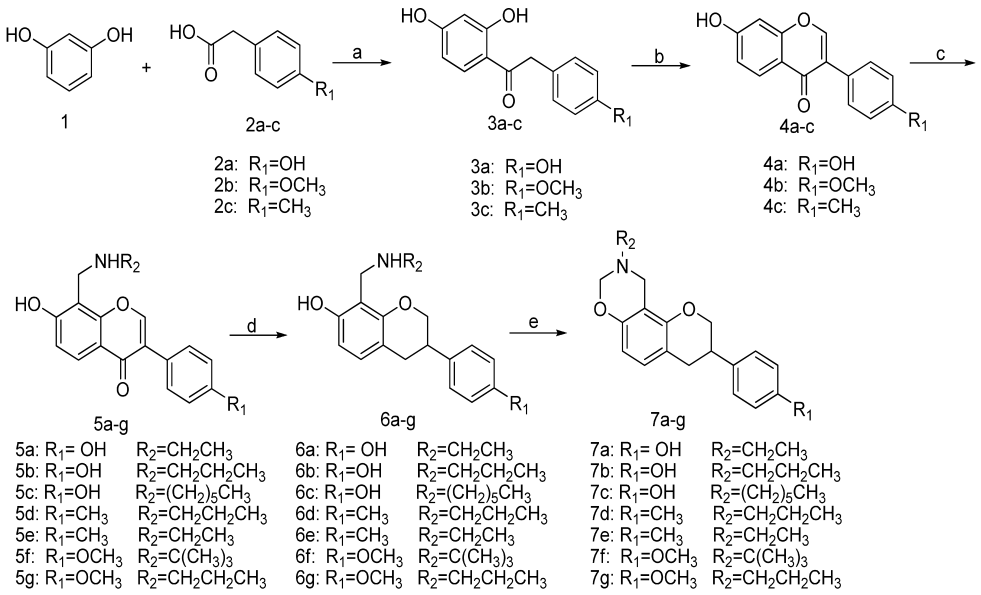


Fig. 2. Four Type Target Compounds

* To whom correspondence should be addressed. e-mail: wangduncn@hotmail.com.

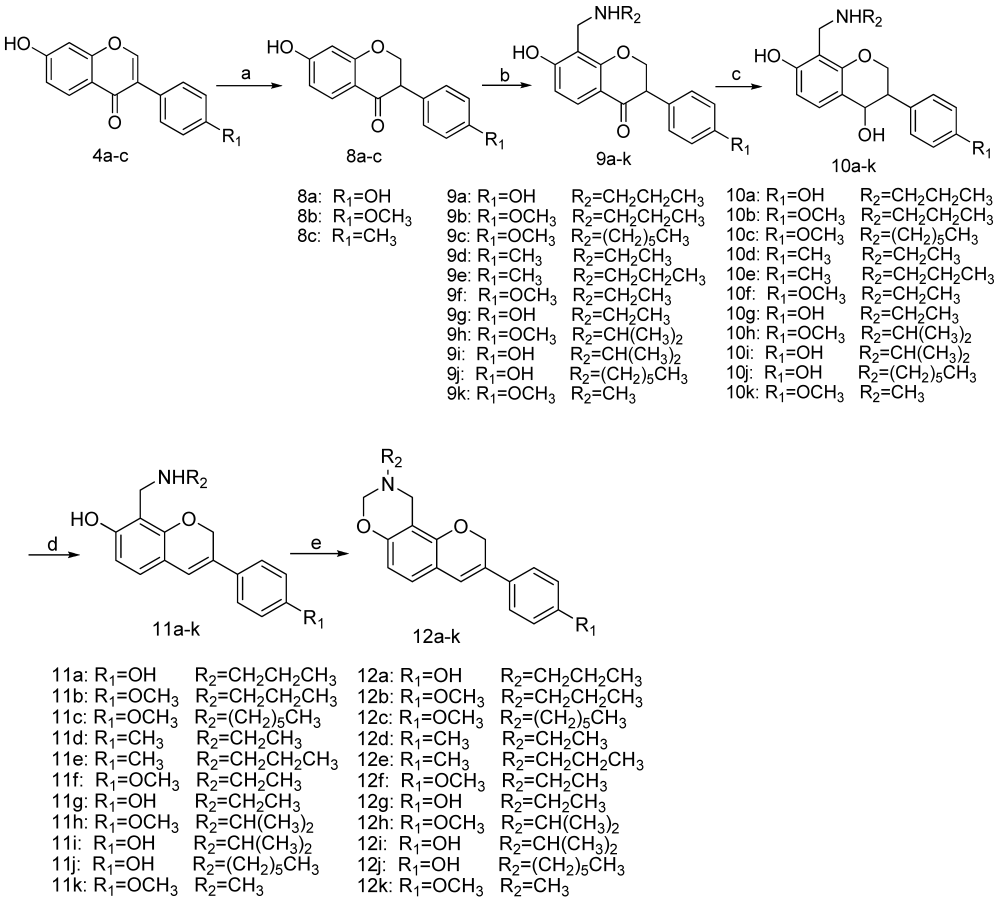


Reaction conditions: (a) $BF_3{\cdot}Et_2O$/80°C; (b) CH_3SO_2CI/DMF, 50–70°C; (c) R_2NH_2, 37% HCHO, 1,4-dioxane, 45–70°C; (d) 10% Pd/C, $HCOONH_4$, CH_3COOH, 110°C; (e) $(HCHO)_n$, KOH, CH_3OH

Chart 1. The Synthesis of Oxazinyl Isoflavonoid Compounds **7a**–**g**

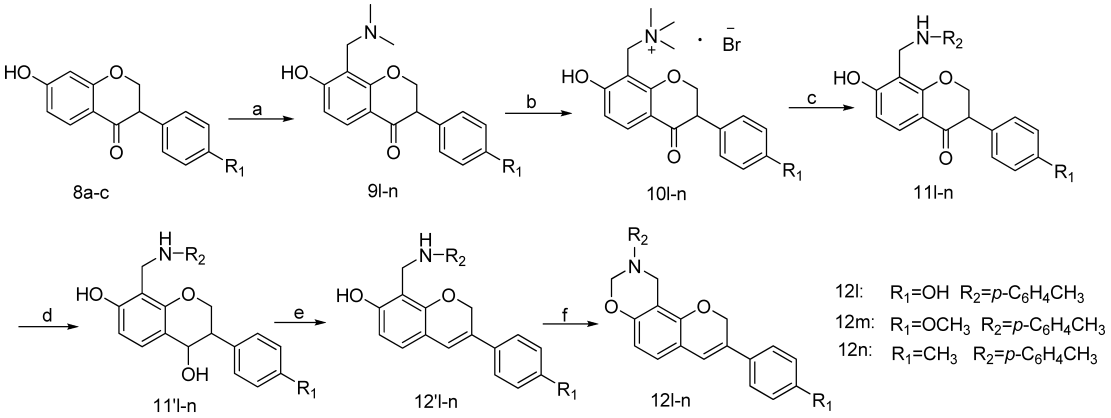
Table 1. *In Vitro* Growth Inhibitory Effects of the Target Compounds **7a**–**18d** in Three Human Cancer Cell Lines

Compd.	R_1	R_2	*In vitro* IC_{50} (μM)		
			HL60	SKOV-3	DU-145
7a	OH	CH_2CH_3	0.123	19.505	22.693
7b	OH	$CH_2CH_2CH_3$	11.86	17.131	41.585
7c	OH	$(CH_2)_5CH_3$	2.185	13.206	7.706
7d	CH_3	$CH_2CH_2CH_3$	4.645	23.155	20.096
7e	CH_3	CH_2CH_3	4.809	27.04	16.953
7f	OCH_3	$C(CH_3)_3$	0.763	8.132	9.113
7g	OCH_3	$CH_2CH_2CH_3$	2.195	16.784	21.591
12a	OH	$CH_2CH_2CH_3$	7.11	23.173	30.108
12b	OCH_3	$CH_2CH_2CH_3$	4.55	21.613	23.500
12c	OCH_3	$(CH_2)_5CH_3$	5.89	31.984	14.379
12d	CH_3	CH_2CH_3	2.614	60.008	39.639
12e	CH_3	$CH_2CH_2CH_3$	1.006	67.25	39.668
12f	OCH_3	CH_2CH_3	5.50	22.270	27.683
12g	OH	CH_2CH_3	5.15	28.554	14.018
12h	OCH_3	$CH(CH_3)_2$	3.04	7.483	9.101
12i	OH	$CH(CH_3)_2$	1.78	11.772	8.030
12j	OH	$(CH_2)_5CH_3$	3.16	16.577	18.395
12k	OCH_3	CH_3	1.859	24.398	20.932
12l	OH	*p*-$C_6H_4CH_3$	8.14	>100	>100
12m	OCH_3	*p*-$C_6H_4CH_3$	17.404	61.102	65.856
12n	CH_3	*p*-$C_6H_4CH_3$	5.616	>100	>100
13a	OH	$CH_2CH_2CH_3$	55.788	93.827	>100
13b	OCH_3	$CH_2CH_2CH_3$	>100	>100	>100
13c	OCH_3	$(CH_2)_5CH_3$	23.81	7.432	58.817
13d	CH_3	CH_2CH_3	52.92	70.893	82.857
13e	CH_3	$CH_2CH_2CH_3$	>100	>100	>100
18a	—	CH_2CH_3	3.52	34.416	27.649
18b	—	$CH_2CH_2CH_3$	1.796	35.896	22.037
18c	—	CH_3	1.569	39.338	23.371
18d	—	$CH(CH_3)_2$	3.121	25.467	11.976
Cisplatin			12.08	32.15	28.42
Phenoxodiol			35.102	30.558	21.03



Reaction conditions: (a) 10% Pd/C, $HCOONH_4$, CH_3OH, 70° (b) R_2NH_2, 37% HCHO, 1,4-dioxane, 45–70°C; (c) $NaBH_4$, CH_3OH; (d) EtOH, HCl·EtOH; (e) $(HCHO)_n$, KOH, CH_3OH

Chart 2. The Synthesis of Oxazinyl Isoflavonoid Compounds **12a**–**k**

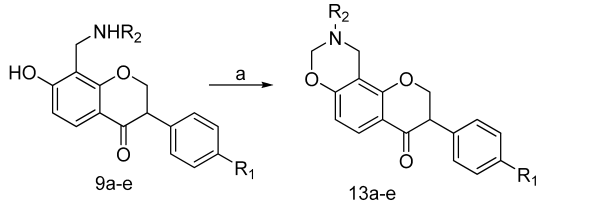


Reaction conditions: (a) $(CH_3)_2NH$, 37% HCHO, 1,4-dioxane, 45–70°C; (b) CH_3COCH_3, CH_3Br, r.t.; (c) $ArNH_2$, CH_3COOH, EtOH, 80°; (d) $NaBH_4$, CH_3OH; (e) EtOH, HCl·$EtOH_2$; (f) $(HCHO)_n$, KOH, CH_3OH

Chart 3. The Synthetic Route of Target Compounds **12l**–**n**

compounds **4a**–**c** with a primary amine and formaldehyde in 1,4-dioxane *via* a Mannich reaction[7] afforded compounds **5a**–**g**, which were converted into their corresponding isoflavans **6a**–**g** *via* transfer hydrogenations with ammonium formate, as the hydrogen donator, in acetic acid. By reacting compounds **6a**–**g** with paraformaldehyde in the presence of potassium hydroxide, compounds of type I, the chromano[8,7-*e*][1,3]oxazine derivatives **7a**–**g**, were obtained, respectively (Chart 1).

The reductions of intermediates **4a**–**c** were performed *via* transfer hydrogenations in methanol to obtain isoflavanones **8a**–**c**,[8] which were treated with a primary amine and formaldehyde to obtain the Mannich products **9a**–**k**. Next, reduction of **9a**–**k** with $NaBH_4$ in methanol conveniently afforded isolavan-4-ols **10a**–**k**, which were dehydrolyzed by HCl/EtOH in ethanol at room temperature to yield compounds **11a**–**k**. Finally, treating **11a**–**k** with paraformaldehyde and potassium hydroxide gave compounds of type II, the desired chromeno[8,7-*e*][1,3]oxazine derivatives **12a**–**k** (Chart 2).



Reaction conditions: (a) $(HCHO)_n$, KOH, CH_3OH

Chart 4. The Synthesis of Oxazinyl Isoflavonoid Compounds **13a**–**e**

Unfortunately, the chromeno[8,7-*e*][1,3]oxazine derivatives **12l**–**n**, bearing the aryl group at the N-9 position, could not be prepared according to the procedure described above. The main reason is that **11l**–**n** can't be prepared directly by Mannich Reaction. As shown in Chart 3, isolavanones **8a**–**c** were instead treated with dimethylamine and formaldehyde to generate Mannich bases **9l**–**n**, which were subsequently quaternized in acetone with CH_3Br at room temperature to achieve quaternary ammonium salts **10l**–**n**. Next, compounds **11l**–**n** were prepared by reacting **10l**–**n** with an arylamine in ethanol and acetic acid as the catalyst. Reducing the carbonyl groups at the C-4 positions of **11l**–**n**, followed by dehydration and cyclization, provided target compounds **12l**–**n** (Chart 3).

Compounds of type III, the chromeno[8,7-*e*][1,3]oxazin-4-ones **13a**–**e**, were prepared by directly reacting **9a**–**e** with paraformaldehyde and potassium hydroxide in methanol while in an ice bath (Chart 4).

Compound **8a** treated with dimethyl sulfate in the presence of sodium bicarbonate provided selectively methylated compound **14**. Treatment of **14** with KBH_4 instead of $NaBH_4$ in methanol provided 7-methoxy-4′-hydroxyisoflavan-4-ol (**15**), which was dehydrolyzed to compound **16**. The reaction time had been shortened and the yields had been improved by using KBH_4 instead of $NaBH_4$ on the basis of the following experimental result. Compound **16** underwent a Mannich reaction, followed by condensation with paraformaldehyde to give compounds of type IV, 6-(chromen-3-yl)-benzo[*e*][1,3]oxazine **18a**–**d** (Chart 5).

Results and Discussion

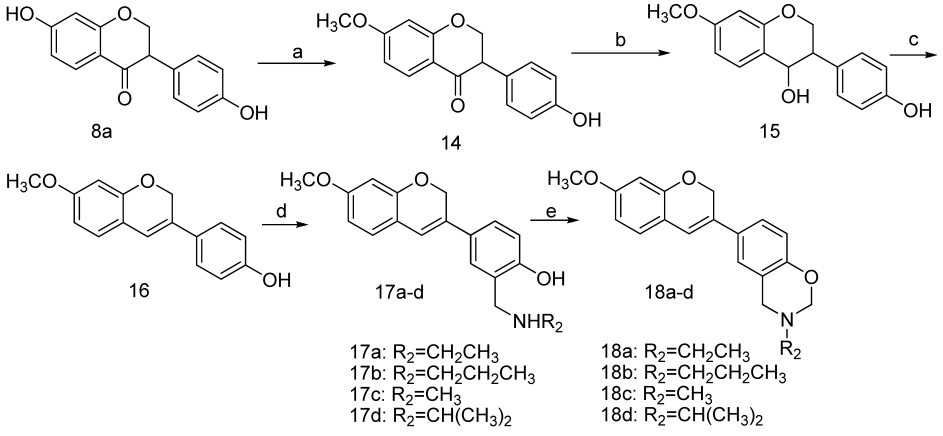
Thirty compounds were synthesized in the current study, and we utilized the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay to determine their IC_{50} *in vitro* growth inhibitory values in a human ovarian cancer cell line (SKOV-3) and human prostate cancer cell line (DU-145). Additionally, the Trypan blue dye exclusion assay was employed to evaluate the growth inhibitory effects of the tested compounds against a human leukemic cell line (HL-60). Cisplatin and parental compound phenoxodiol were both used as positive controls. The ability of these new analogs to inhibit the growth of cancer cell lines is summarized in Table 1.

In general, with modifications to the A ring, both isoflavane derivatives **7a**–**g** and isoflavene derivatives **12a**–**k** showed increased activities compared with their parent compound, phenoxodiol. Compounds **7f**, **12h** and **12i** showed the best activities with IC_{50} values in the micromolar range (1–10 μM), which may suggest that the increase in the steric hindrance of the aliphatic alkyl substituent on the N-position of the oxazinyl ring is favorable for activity. This finding was further confirmed by **7c**, which bore an *n*-hexyl group on the oxazinyl ring. The fact that compounds **12d** and **12e** diminished the activity revealed that the presence of a hydroxy or methoxy group on the *para*-position of the C ring was essential for optimum biological activity. This finding is consistent with the author's previous hypothesis.

The *N*-phenyl substituted isoflavene derivatives **12l**–**n** showed remarkably decreased activities, while compound **12n** almost lost activity completely (>100 μM), which may imply that an aryl substitution on the oxazinyl ring is not well tolerated.

Isoflavanone derivatives **13a**–**e** were generally inactive, while **13b** and **13e** had the lowest activities (IC_{50}>100 μM). These results demonstrated that the structural skeleton of isoflavanone had negative effects.

Compounds **18a**–**d** with modifications on the C ring exhibited comparable activities to phenoxodiol but were less potent than **12a**–**k**. These two types of compounds were of the same structural skeleton, but the positions of their oxazinyl ring were different, suggesting that integrating the oxazinyl functionality with the A ring of isoflavene is better favored. Of compounds **18a**–**d**, **18d**, whose oxazinyl ring was substituted



Reaction conditions: (a) $(CH_3)_2SO_4$, $NaHCO_3$, $(CH_3)_2CO$; (b) KBH_4, CH_3OH; (c) EtOH, HCl·EtOH; (d) R_2NH_2, 37% HCHO, acetic acid, 1,4-dioxane, 45–70°C; (e) $(HCHO)_n$, KOH, CH_3OH

Chart 5. The synthesis of Oxazinyl Isoflavonoid Compounds **18a**–**d**

by an isopropyl group, displayed better potency than the other three compounds. This result was consistent with those we observed in compounds **12a**–**k**.

Conclusions

In this study, a series of novel isoflavoids containing protected phenolic groups with oxazinyl moieties was synthesized, and each compound was evaluated for its *in vitro* anti-tumor activities. These compounds showed increased stabilities compared to the parental compound, phenoxodiol. Additionally, the oxazinyl isoflavane and oxazinyl isoflavene derivatives exhibited potent growth inhibitory activities against the SKOV-3, DU-145 and HL-60 cell lines. Specifically, compound **7f** showed the most potent inhibition activities with IC_{50} values of 0.763, 8.132 and 9.113 μmol, respectively. This compound was followed by compound **12h**, whose IC_{50} values were 3.04, 7.483 and 9.101 μmol, respectively. We observed that both of the above compounds had aliphatic alkyl groups with steric hindrances at the N-atoms of their oxazinyl rings. Overall, the information from this study may be helpful for the design and synthesis of isoflavoid derivatives with stronger activities and better stabilities.

Experimental

All the reagents and solvents were purchased from common commercial suppliers and used without further purification. Nuclear magnetic resonance spectra were recorded in $CDCl_3$ or *d*-DMSO solutions on a Bruker AX 300 spectrometer. Chemical shifts were reported in δ (ppm) relative to the internal standard of tetramethylsilane (TMS), and *J* values were reported in Hz. Mass spectra were taken on an Agilent 1100 LC-MS electrospray/ion trap instrument in positive and negative ion modes. Compounds **3a**–**c**, **4a**–**c**, and **8a**–**c** were synthesized according to the literature.[6,8,9]

3-(4-R_1-Phenyl)-9-R_2-2,3,4,8,9,10-hexahydrochromeno-[8,7-*e*][1,3]oxazines (7a–g) A primary amine (20.7 mmol) and a 37% formaldehyde solution (1.4 g, 16.6 mmol) were dissolved in 1,4-dioxane (1 mL) at 45°C and stirred for 15 min, and then the resulting mixture was added to a solution mixture of **4a**–**c** (13.8 mmol) in 1,4-dioxane (35 mL). The mixture was heated at 70°C for 2–4 h and then concentrated *in vacuo*. The residue was collected under suction filtration and dried to afford compounds **5a**–**g**.

Next, ammonium formate (3.97 g, 63 mmol) and 10% Pd/C (1.5 g), followed by solids **5a**–**g** (10.0 mmol), were added to acetic acid (45 mL). The suspension was heated to 110°C for 45 min. After filtering, the filtrate was concentrated to remove a majority of the solvent, and subsequently poured into ice-cold water (100 mL) and extracted with chloroform (2×60 mL). The collected organic layers were dried over Na_2SO_4, and the solvent was removed under reduced pressure to give **6a**–**g**.

Finally, compounds **6a**–**g** (4.0 mmol) were dissolved in methanol (2.0 mL), and the methanolic solution was added to paraformaldehyde (0.36 g, 12.0 mmol) and potassium hydroxide (0.02 g, 0.4 mmol), which were stirred at room temperature. After stirring, a white precipitate was collected to afford compounds **7a**–**g**.

7a: Yield 56%; MS $[M+H]^+$ (*m/z*): 312.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.20 (3H, t, *J*=7.2 Hz, CH_3), 2.81 (2H, q, *J*=7.2 Hz, NCH_2CH_3), 2.91 (2H, m, $PhCH_2CH$), 3.11 (1H, m, CH_2CHCH_2O), 3.94 (2H, s, $PhCH_2N$), 3.94–4.30 (2H, m, OCH_2CH), 4.83 (2H, s, OCH_2N), 6.38 (1H, d, *J*=8.4 Hz, H6), 6.84 (1H, d, *J*=8.4 Hz, H5), 6.80 (2H, d, *J*=8.4 Hz, H3′, H5′), 7.09 (2H, d, *J*=8.4 Hz, H2′, H6′).

7b: Yield 62%; MS $[M+H]^+$ (*m/z*): 326.2; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.93 (3H, t, *J*=7.5 Hz, CH_3), 1.61 (2H, m, CH_2CH_3), 2.72 (2H, t, *J*=7.2 Hz, NCH_2CH_2), 2.94 (2H, m, $PhCH_2CH$), 3.13 (1H, m, CH_2CHCH_2O), 3.93 (2H, s, $PhCH_2N$), 3.93–4.30 (2H, m, OCH_2CH), 4.82 (2H, s, OCH_2N), 6.38 (1H, d, *J*=8.4 Hz, H6), 6.84 (1H, d, *J*=8.4 Hz, H5), 6.82 (2H, d, *J*=8.4 Hz, H3′, H5′), 7.10 (2H, d, *J*=8.4 Hz, H2′, H6′).

7c: Yield 58%; MS$[M+H]^+$ (*m/z*): 368.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (3H, t, *J*=6.6 Hz, CH_3), 1.30–1.56 (8H, m, $CH_3(CH_2)_4$), 2.75 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 2.91 (2H, m, $PhCH_2CH$), 3.12 (1H, m, CH_2CHCH_2O), 3.91 (2H, s, $PhCH_2N$), 3.91–4.31 (2H, m, OCH_2CH), 4.81 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.85 (1H, d, *J*=8.4 Hz, H5), 6.81 (2H, d, *J*=7.8 Hz, H3′, H5′), 7.10 (2H, d, *J*=7.8 Hz, H2′, H6′).

7d: Yield 72%; MS $[M+H]^+$ (*m/z*): 324.3; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.93 (3H, t, *J*=7.2 Hz, CH_3), 1.59 (2H, m, CH_2CH_3), 2.34 (3H, s, $PhCH_3$), 2.70 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 2.91 (2H, m, $PhCH_2CH$), 3.14 (1H, m, CH_2CHCH_2O), 3.92 (2H, s, $PhCH_2N$), 3.97–4.34 (2H, m, OCH_2CH), 4.81 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.84 (1H, d, *J*=8.1 Hz, H5), 7.12 (2H, d, *J*=8.4 Hz, H3′, H5′), 7.17 (2H, d, *J*=8.4 Hz, H2′, H6′).

7e: Yield 76%; MS $[M+H]^+$ (*m/z*): 310.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.19 (3H, t, *J*=7.2 Hz, CH_3), 2.35 (3H, s, $PhCH_3$), 2.80 (2H, q, *J*=7.2 Hz, NCH_2CH_3), 2.94 (2H, m, $PhCH_2CH$), 3.15 (1H, m, CH_2CHCH_2O), 3.93 (2H, s, $PhCH_2N$), 3.98–4.33 (2H, m, OCH_2CH), 4.83 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.85 (1H, d, *J*=8.4 Hz, H5), 7.13 (2H, d, *J*=8.4 Hz, H3′, H5′), 7.17 (2H, d, *J*=8.1 Hz, H2′, H6′).

7f: Yield 70%; MS $[M+H]^+$ (*m/z*): 354.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.22 (9H, s, $C(CH_3)_3$), 2.92 (2H, m, $PhCH_2CH$), 3.16 (1H, m, CH_2CHCH_2O), 3.81 (3H, s, $PhOCH_3$), 4.00 (2H, s, $PhCH_2N$), 3.95–4.34 (2H, m, OCH_2CH), 4.93 (2H, s, OCH_2N), 6.35 (1H, d, *J*=8.1 Hz, H6), 6.82 (1H, d, *J*=8.4 Hz, H5), 6.89 (2H, d, *J*=8.7 Hz, H3′, H5′), 7.17 (2H, d, *J*=8.7 Hz, H2′, H6′).

7g: Yield 75%; MS $[M+H]^+$ (*m/z*): 325.9; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.93 (3H, t, *J*=7.5 Hz, CH_3), 1.60 (2H, m, CH_2CH_3), 2.71 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 2.93 (2H, m, $PhCH_2CH$), 3.14 (1H, m, CH_2CHCH_2O), 3.81 (3H, s, $PhOCH_3$), 3.91 (2H, s, $PhCH_2N$), 3.95–4.32 (2H, m, OCH_2CH), 4.81 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.1 Hz, H6), 6.84 (1H, d, *J*=8.4 Hz, H5), 6.89 (2H, d, *J*=8.7 Hz, H3′, H5′), 7.16 (2H, d, *J*=8.7 Hz, H2′, H6′).

3-(4-R1-Phenyl)-9-R_2-2,8,9,10-tetrahydrochromeno[8,7-*e*][1,3]oxazines (12a–n) Compounds **9a**–**k** were obtained using compounds **8a**–**c** as the starting materials in the Mannich reaction described in Section "Synthesis". Next, a solid mixture of compounds **9a**–**k** (8.0 mmol) was dissolved in methanol (160 mL) and treated with $NaBH_4$ (3.1 g, 80 mmol) for 48 h at room temperature. The reaction was quenched with a saturated ammonium chloride solution (12.5 mL) and then was poured into ice water (500 mL). The solution was extracted with ethyl acetate (2×100 mL), and the ethyl acetate layers were dried with Na_2SO_4 and concentrated under reduced pressure to give solids **10a**–**k**. Compounds **10a**–**k** (3.0 mmol) were dehydrated by treating with HCl/EtOH (5.0 mL, 4.5 mmol), and thus, a faint yellow precipitate, containing compounds

11a–**k**, was collected under suction filtration. Title compounds **12a**–**k** were prepared by reacting **11a**–**k** (1.5 mmol) with paraformaldehyde (0.14 g, 4.5 mmol) in the presence of potassium hydroxide at room temperature in methanol.

12a: Yield 87%; MS $[M+H]^+$ (*m/z*): 323.9; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 0.94 (3H, t, *J*=7.2 Hz, CH_3), 1.63 (2H, m, CH_2CH_3), 2.72 (2H, t, *J*=7.4 Hz, NCH_2CH_2), 3.96 (2H, s, $PhCH_2N$), 4.84 (2H, s, OCH_2C), 5.10 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.1 Hz, H6), 6.63 (1H, s, PhCH=C), 6.84 (2H, d, *J*=8.7 Hz, H3′, H5′), 6.85 (1H, d, *J*=8.1 Hz, H5), 7.28 (2H, d, *J*=8.7 Hz, H2′, H6′).

12b: Yield 89%; MS $[M+H]^+$ (*m/z*): 338.1; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 0.94 (3H, t, *J*=7.35 Hz, CH_3), 1.60 (2H, m, CH_2CH_3), 2.71 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 3.83 (3H, s, $PhOCH_3$), 3.95 (2H, s, $PhCH_2N$), 4.84 (2H, s, OCH_2C), 5.12 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.28 Hz, H6), 6.65 (1H, s, PhCH=C), 6.85 (1H, d, *J*=8.31 Hz, H5), 6.91 (2H, d, *J*=8.73 Hz, H3′, H5′), 7.34 (2H, d, *J*=8.73 Hz, H2′, H6′).

12c: Yield 90%; MS $[M+H]^+$ (*m/z*): 380.2; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 0.89 (3H, t, *J*=6.6 Hz, CH_3), 1.30–1.56 (8H, m, $CH_3(CH_2)_4$), 2.73 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 3.83 (3H, s, $PhOCH_3$), 3.95 (2H, s, $PhCH_2N$), 4.84 (2H, s, OCH_2C), 5.12 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.65 (1H, s, PhCH=C), 6.86 (1H, d, *J*=8.1 Hz, H5), 6.92 (2H, d, *J*=9.0 Hz, H3′, H5′), 7.34 (2H, d, *J*=8.8 Hz, H2′, H6′).

12d: Yield 90%; MS $[M+H]^+$ (*m/z*): 308.1; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 1.19 (3H, t, *J*=7.2 Hz, CH_3), 2.36 (3H, s, $PhCH_3$), 2.81 (2H, q, *J*=6.9 Hz, NCH_2CH_3), 3.97 (2H, s, $PhCH_2N$), 4.85 (2H, s, OCH_2C), 5.14 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.70 (1H, s, PhCH=C), 6.86 (1H, d, *J*=8.4 Hz, H5), 7.18 (2H, d, *J*=8.1 Hz, H3′, H5′), 7.30 (2H, d, *J*=7.8 Hz, H2′, H6′).

12e: Yield 89%; MS $[M+H]^+$ (*m/z*): 321.9; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 0.94 (3H, t, *J*=7.2 Hz, CH_3), 1.61 (2H, m, CH_2CH_3), 2.36 (3H, s, $PhCH_3$), 2.71 (2H, q, *J*=7.5 Hz, NCH_2CH_2), 3.95 (2H, s, $PhCH_2N$), 4.84 (2H, s, OCH_2C), 5.13 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.71 (1H, s, PhCH=C), 6.86 (1H, d, *J*=8.1 Hz, H5), 7.18 (2H, d, *J*=8.1 Hz, H3′, H5′), 7.30 (2H, d, *J*=8.1 Hz, H2′, H6′).

12f: Yield 87%; MS $[M+H]^+$ (*m/z*): 323.9; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 1.19 (3H, t, *J*=7.2 Hz, CH_3), 2.81 (2H, q, *J*=7.2 Hz, NCH_2CH_3), 3.83 (3H, s, $PhOCH_3$), 3.97 (2H, s, $PhCH_2N$), 4.86 (2H, s, OCH_2C), 5.12 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.4 Hz, H6), 6.65 (1H, s, PhCH=C), 6.86 (1H, d, *J*=8.4 Hz, H5), 6.92 (2H, d, *J*=9.0 Hz, H3′, H5′), 7.35 (2H, d, *J*=8.8 Hz, H2′, H6′).

12g: Yield 86%; MS $[M+H]^+$ (*m/z*): 310.1; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 1.08 (3H, t, *J*=7.2 Hz, CH_3), 2.68 (2H, q, *J*=7.2 Hz, NCH_2CH_3), 3.84 (2H, s, $PhCH_2N$), 4.80 (2H, s, OCH_2C), 5.10 (2H, s, OCH_2N), 6.31 (1H, d, *J*=8.4 Hz, H6), 6.76 (1H, s, PhCH=C), 6.78 (2H, d, *J*=8.7 Hz, H3′, H5′), 6.89 (1H, d, *J*=8.1 Hz, H5), 7.35 (2H, d, *J*=8.7 Hz, H2′, H6′).

12h: Yield 87%; MS $[M+H]^+$ (*m/z*): 338.2; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 1.17 (6H, d, *J*=6.6 Hz, $CH(CH_3)_2$), 3.11 (H, m, $NCH(CH_3)_2$), 3.83 (3H, s, $PhOCH_3$), 4.01 (2H, s, $PhCH_2N$), 4.93 (2H, s, OCH_2C), 5.12 (2H, s, OCH_2N), 6.35 (1H, d, *J*=8.25 Hz, H6), 6.65 (1H, s, PhCH=C), 6.84 (1H, d, *J*=8.27 Hz, H5), 6.91 (2H, d, *J*=8.8 Hz, H3′, H5′), 7.35 (2H, d, *J*=8.8 Hz, H2′, H6′).

12i: Yield 86%; MS $[M+H]^+$ (*m/z*): 324.2; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 1.18 (6H, d, *J*=6.4 Hz, $CH(CH_3)_2$), 3.10 (1H, m, $NCH(CH_3)_2$), 4.02 (2H, s, $PhCH_2N$), 4.93 (2H, s, OCH_2C), 5.11 (2H, s, OCH_2N), 6.36 (1H, d, *J*=8.1 Hz, H6), 6.63 (1H, s, PhCH=C), 6.83 (2H, d, *J*=8.7 Hz, H3′, H5′), 6.83 (1H, d, *J*=8.1 Hz, H5), 7.29(2H, d, *J*=8.7 Hz, H2′, H6′).

12j: Yield 87%; MS $[M+H]^+$ (*m/z*): 365.8; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 0.87 (3H, t, *J*=6.6 Hz, CH_3), 1.28–1.58 (8H, m, $CH_3(CH_2)_4$), 2.73 (2H, t, *J*=7.5 Hz, NCH_2CH_2), 3.95 (2H, s, $PhCH_2N$), 4.84 (2H, s, OCH_2C), 5.11 (2H, s, OCH_2N), 6.37 (1H, d, *J*=8.1 Hz, H6), 6.64 (1H, s, PhCH=C), 6.84 (2H, d, *J*=8.4 Hz, H3′, H5′), 6.85 (1H, d, *J*=8.4 Hz, H5), 7.29 (2H, d, *J*=8.7 Hz, H2′, H6′).

12k: Yield 88%; MS $[M+H]^+$ (*m/z*): 310.2; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 2.62 (3H, s, NCH_3), 3.83 (3H, s, $PhOCH_3$), 3.93 (2H, s, $PhCH_2N$), 4.77 (2H, s, OCH_2C), 5.12 (2H, s, OCH_2N), 6.39 (1H, d, *J*=8.4 Hz, H6), 6.65 (1H, s, PhCH=C), 6.87 (1H, d, *J*=8.4 Hz, H5), 6.93 (2H, d, *J*=8.8 Hz, H3′, H5′), 7.34 (2H, d, *J*=8.8 Hz, H2′, H6′).

Dimethylamine (24 mmol) and a 37% formaldehyde solution (1.56 g, 19.0 mmol) were dissolved in 1,4-dioxane (1 mL) and stirred at 45°C for 15 min, and then the reaction was added to a solution mixture of compounds **8a**–**c** (16 mmol) in 1,4-dioxane (40 mL). The resulting mixture was stirred and heated at 70°C for 30 min, and then it was distilled under reduced pressure until crystals began to form. Compounds **9l**–**n** were collected on a filter and subsequently quaternized by CH_3Br in acetone to give compounds **10l**–**n**. Next, compounds **11l**–**n** were prepared by treating the solid containing compounds **10l**–**n** (11.7 mmol) with an arylamine (18 mmol) in ethanol and using acetic acid as the catalyst. Compounds **11l**–**n** (7.4 mmol) in methanol (60 mL) were reduced to isoflavan-4-ola **11′l**–**n** when treated with $NaBH_4$ (3.1 g, 80 mmol) for 48 h at room temperature, which were dehydrated by reacting with HCl/EtOH (5.0 mL, 4.5 mmol, 0.9 mol/L) to yield a solid containing compounds **12′l**–**n**. Lastly, compounds **12′l**–**n** were condensed with paraformaldehyde (0.14 g, 4.5 mmol) with potassium hydroxide as a catalyst to give title compounds **12l**–**n**.

12l: Yield 86%; MS $[M+H]^+$ (*m/z*): 372.2; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 2.19 (3H, s, $PhCH_3$), 4.51 (2H, s, $PhCH_2N$), 5.13 (2H, s, OCH_2C), 5.36 (2H, s, OCH_2N), 6.31 (1H, d, *J*=8.1 Hz, H6), 6.77 (1H, s, PhCH=C), 6.80 (2H, d, *J*=8.1 Hz, H3′, H5′), 6.89 (1H, d, *J*=8.4 Hz, H5), 6.99–7.07 (4H, d×2, NPh), 7.35 (2H, d, *J*=8.4 Hz, H2′, H6′).

12m: Yield 88%; MS $[M+H]^+$ (*m/z*): 386.1; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 2.26 (3H, s, $PhCH_3$), 3.83 (3H, s, $PhOCH_3$), 4.54 (2H, s, $PhCH_2N$), 5.13 (2H, s, OCH_2C), 5.30 (2H, s, OCH_2N), 6.38 (1H, d, *J*=8.1 Hz, H6), 6.63 (1H, s, PhCH=C), 6.84 (1H, d, *J*=7.8 Hz, H5), 6.91 (2H, d, *J*=8.7 Hz, H3′, H5′), 7.02–7.09 (4H, NPh), 7.34 (2H, d, *J*=8.7 Hz, H2′, H6′).

12n: Yield 86%; MS $[M+H]^+$ (*m/z*): 369.8; ^{1}H-NMR (300 MHz, $CDCl_3$) *δ*: 2.26 (3H, s, $PhCH_3$), 2.34 (3H, s, $PhCH_3$), 4.55 (2H, s, $PhCH_2N$), 5.14 (2H, s, OCH_2C), 5.30 (2H, s, OCH_2N), 6.38 (1H, d, *J*=8.4 Hz, H6), 6.69 (1H, s, PhCH=C), 6.85 (1H, d, *J*=8.4 Hz, H5), 7.02–7.09 (4H, d×2, NPh), 7.15 (2H, d, *J*=8.1 Hz, H3′, H5′), 7.30 (2H, d, *J*=8.1 Hz, H2′, H6′).

3-(4-Methoxyphenyl)-9-methyl-2,3,9,10-tetrahydrochromeno[8,7-*e*][1,3]oxazin-4(8*H*)-on-es (13a–e) A methanolic solution of paraformaldehyde (0.72 g, 24.0 mmol) and potassium hydroxide platelet was added to the solution mixture of compounds **9a**–**e** (8.0 mmol) dissolved in methanol

(2.0 mL). Target compounds **13a–e** were separated, filtered and dried under vacuum filtration.

13a: Yield 87%; MS $[M+H]^+$ (*m/z*): 340.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.94 (3H, t, J=7.2 Hz, CH_3), 1.60 (2H, m, CH_2CH_3), 2.69 (2H, t, J=7.2 Hz, NCH_2CH_2), 3.95 (2H, s, $PhCH_2N$), 3.87 (1H, m, PhCH), 4.60 (2H, m, OCH_2C), 4.91 (2H, s, OCH_2N), 6.50 (1H, d, J=8.7 Hz, H6), 6.72 (2H, d, J=8.4 Hz, H3′, H5′), 7.06 (2H, d, J=8.1 Hz, H2′, H6′), 7.76 (1H, d, J=8.7 Hz, H5).

13b: Yield 90%; MS $[M+H]^+$ (*m/z*): 354.2; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.95 (3H, t, J=7.2 Hz, CH_3), 1.61 (2H, m, CH_3CH_2), 2.71 (2H, t, J=7.2 Hz, NCH_2CH_2), 3.80 (3H, s, $PhOCH_3$), 3.89 (1H, m, PhCH), 3.97 (2H, s, $PhCH_2N$), 4.62 (2H, m, OCH_2C), 4.91 (2H, s, OCH_2N), 6.50 (1H, d, J=8.7 Hz, H6), 6.89 (2H, d, J=8.7 Hz, H3′, H5′), 7.19 (2H, d, J=8.7 Hz, H2′, H6′), 7.77 (1H, d, J=9.0 Hz, H5).

13c: Yield 89%; MS $[M+H]^+$ (*m/z*): 395.9; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.88 (3H, t, J=6.9 Hz, CH_3), 1.31–1.58 (8H, m, $CH_3(CH_2)_4$), 2.72 (2H, t, J=6.9 Hz, NCH_2CH_2), 3.79 (3H, s, $PhOCH_3$), 3.88 (1H, m, PhCH), 3.95 (2H, s, $PhCH_2N$), 4.62 (2H, m, OCH_2C), 4.90 (2H, s, OCH_2N), 6.50 (1H, d, J=8.7 Hz, H6), 6.89 (2H, d, J=8.7 Hz, H3′, H5′), 7.19 (2H, d, J=8.7 Hz, H2′, H6′), 7.77 (1H, d, J=9.0 Hz, H5).

13d: Yield 88%; MS $[M+H]^+$ (*m/z*): 323.8; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.19 (3H, t, J=7.2 Hz, CH_3), 2.32 (3H, s, $PhCH_3$), 2.79 (2H, q, J=7.2 Hz, NCH_2CH_3), 3.80 (3H, s, $PhOCH_3$), 3.89 (1H, m, PhCH), 3.97 (2H, s, $PhCH_2N$), 4.62 (2H, m, OCH_2C), 4.91 (2H, s, OCH_2N), 6.50 (1H, d, J=8.7 Hz, H6), 6.89 (2H, d, J=8.7 Hz, H3′, H5′), 7.19 (2H, d, J=8.7 Hz, H2′, H6′), 7.77 (1H, d, J=9.0 Hz, H5).

13e: Yield 90%; MS $[M+H]^+$ (*m/z*): 386.2; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.94 (3H, t, J=7.35 Hz, CH_3), 1.62 (2H, m, CH_2CH_3), 2.33 (3H, s, $PhCH_3$), 2.69 (2H, t, J=7.5 Hz, NCH_2CH_2), 3.80 (3H, s, $PhOCH_3$), 3.89 (1H, m, PhCH), 4.00 (2H, s, $PhCH_2N$), 4.64 (2H, m, OCH_2C), 4.99 (2H, s, OCH_2N), 6.47 (1H, d, J=8.7 Hz, H6), 7.15 (4H, s, H3′, H5′, H2′, H6′), 7.77 (1H, d, J=8.7 Hz, H5).

6-(7-Methoxy-2*H*-chromen-3-yl)-3-R$_2$-3,4-dihydro-2*H*-benzo[*e*][1,3]oxazines (18a–d) Dihydrodaidzein was synthesized according to patent WO9808503A1. Briefly, to a mixture of dihydrodaidzein (4.0 g, 15.7 mmol) and sodium bicarbonate (6.0 g) in acetone (100 mL) was added dimethyl sulfate (2.02 g, 16 mmol) dropwise under stirring at 60°C, and then the reaction was refluxed for 20 h. After filtration, the filtrate was poured into water (400 mL) with stirring to give 3-(4-hydroxyphenyl)-7-methoxychroman-4-one (3.78 g, 14 mmol) as a white solid, which was dissolved in methanol (50 mL) and treated with KBH_4 (3.02 g, 56 mmol) at room temperature for 30 min. The reaction was quenched with a saturated ammonium chloride solution (20 mL) and then ice water (250 mL); compound **15** (3.10 g, 11 mmol) precipitated as a white solid. Compound **15** was treated with HCl/EtOH (34.1 mL, 0.5 mol/L) to make the hydroxide radical at the C-4 position. The reaction mixture was left in the refrigerator overnight to crystallize, and compound **16** (2.02 g, 8 mmol) was collected under suction filtration. Next, treatment of compound **16** with a primary amine (12 mmol) and a 37% formaldehyde solution (0.78 g, 9.6 mmol) in 1,4-dioxane yielded compounds **17a–d**, which were reacted with paraformaldehyde (0.47 g, 16 mmol) and potassium hydroxide (0.04 g, 0.8 mmol) in methanol (2 mL) at room temperature to obtain title compounds **18a–d**.

18a: Yield 70%; MS $[M+H]^+$ (*m/z*): 324.2; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.18 (3H, t, J=7.2 Hz, CH_3), 2.81 (2H, q, J=7.2 Hz, NCH_2CH_3), 3.79 (3H, s, $PhOCH_3$), 4.02 (2H, s, $PhCH_2N$), 4.90 (2H, s, OCH_2C), 5.08 (2H, s, OCH_2N), 6.43 (1H, s, H8′), 6.47 (1H, dd, J=8.1, 2.7 Hz, H6′), 6.65 (1H, s, H4′), 6.77 (H, d, J=8.4 Hz, H5′), 6.96 (1H, s, H5), 7.00 (1H, d, J=8.7 Hz, H8), 7.19 (1H, dd, J=8.7, 2.1 Hz, H7).

18b: Yield 69%; MS $[M+H]^+$ (*m/z*): 338.1; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.93 (3H, t, J=7.8 Hz, CH_3), 1.59 (2H, m, CH_2CH_3), 2.72 (2H, t, J=7.8 Hz, NCH_2CH_2), 3.79 (3H, s, $PhOCH_3$), 4.01 (2H, s, $PhCH_2N$), 4.89 (2H, s, OCH_2C), 5.09 (2H, s, OCH_2N), 6.44 (1H, s, H8′), 6.47 (1H, dd, J=8.4, 2.4 Hz, H6′), 6.66 (1H, s, H4′), 6.78 (H, d, J=8.4 Hz, H5′), 6.97 (1H, d, J=8.4 Hz, H8), 7.00 (1H, s, H5), 7.19 (1H, dd, J=8.4, 1.2 Hz, H7).

18c: Yield 75%; MS $[M+H]^+$ (*m/z*): 309.9; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.62 (1H, s, NCH_3), 3.79 (3H, s, $PhOCH_3$), 3.98 (2H, s, $PhCH_2N$), 4.82 (2H, s, OCH_2C), 5.09 (2H, s, OCH_2N), 6.44 (1H, d, J=2.1 Hz, H8′), 6.47 (1H, dd, J=8.4, 2.4 Hz, H6′), 6.66 (1H, s, H4′), 6.80 (H, d, J=8.7 Hz, H8), 6.98 (1H, d, J=8.1 Hz, H5′), 7.01 (1H, d, J=1.8 Hz, H5), 7.21 (1H, dd, J=8.4, 2.1 Hz, H7).

18d: Yield 65%; MS $[M+H]^+$ (*m/z*): 337.8; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.17 (6H, d, J=6.3 Hz, $CH(CH_3)_2$), 3.08–3.16 (1H, m, J=6.3 Hz, $NCH(CH_3)_2$), 3.79 (3H, s, $PhOCH_3$), 4.09 (2H, s, $PhCH_2N$), 4.98 (2H, s, OCH_2C), 5.09 (2H, s, OCH_2N), 6.43 (1H, d, J=2.1 Hz, H8′), 6.47 (1H, dd, J=8.1, 2.4 Hz, H6′), 6.66 (1H, s, H4′), 6.76 (H, d, J=8.7 Hz, H8), 6.97 (1H, d, J=8.1 Hz, H5′), 7.01 (1H, d, J=2.1 Hz, H5), 7.17 (1H, dd, J=8.4, 2.1 Hz, H7).

Determination of *in Vitro* Anticancer Activities against SKOV-3 and DU-145 Cell Lines Using a MTT Assay The three cell lines used in this study were cultured in RPMI1640 medium containing sodium bicarbonate (2.0 g/L) supplemented with 10% (v/v) heat-inactivated (57°C, 40 min) fetal calf serum (FCS), streptomycin (100 μg/mL), penicillin (100 IU/mL), and L-glutamine (4 μmol/mL); all of these substances were obtained from Serva (Heidelberg, FRG). All of the cell lines were free of pathogenic contaminations during the experiments and were grown as monolayers in a humidified atmosphere (5% CO_2/95% air) at 37°C. Exponentially grown cells were plated at 5×10^4 cells/cm^{-2} into 96-well plates and incubated for 24 h. Next, the cells were treated with serial dilutions of the tested compounds at the following final concentrations: 100, 30, 10, 3 and 1 μM. Stock solutions of the compounds were initially dissolved in 20% dimethyl sulfoxide (DMSO) and further diluted with fresh complete medium.

After 72 h of incubation at 37°C, the medium was removed and 20 μL of MTT reagent (5 mg/mL) in serum-free medium was added to each well. The plates were incubated at 37°C for 4 h. At the end of the incubation period, the medium was removed and pure DMSO (150 μL) was added to each well. The metabolized MTT products dissolved in DMSO were quantified by reading their absorbances at 570 nm using a spectrophotometer. IC_{50} values (the concentrations of the compounds that caused 50% cell growth inhibition) were calculated with data processing software (SPSS).

Determinations of *in Vitro* Anticancer Activities against the HL-60 Cell Line by the Trypan Blue Exclusion Assay Exponentially grown cells were plated at 4×10^4 cells/cm^{-2} into

24-well plates, incubated for 24 h, and then the compounds were added to obtain the following final concentrations: 100, 30, 10, 3 and 1 μM. The vehicle cells were exposed to culture medium containing 0.1% DMSO. After incubation for 72 h at 37°C, the cells were collected and suspended in 0.4% Trypan blue. The number of viable cells was counted using a hemocytometer, and IC_{50} values were calculated using data processing software (SPSS).

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